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Vol. XLVI *June, 1924* *No. 6*

BIOLOGICAL BULLETIN

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

I. Male Secondary Sexual Characters in Females of *Cambarus propinquus*.

C. L. TURNER,

Zoölogical Laboratory, Beloit College.

During the early spring of 1922 the writer found five specimens of *Cambarus propinquus* which had both male and female secondary sexual characters. Two of the specimens were destroyed in dissecting the internal organs, in examining the annulus for sperm and in separating the appendages preparatory to drawing them. A review of the literature revealed that the condition is extremely rare. Many thousands of specimens have come under the close scrutiny of Faxon, Hagen, Hay, Ortmann and others and only thirteen cases in the genus *Cambarus* have been recorded in which male and female secondary sexual characters have been found to exist in the same individuals. The finding of the five specimens seems all the more remarkable because all were taken within a short distance of each other in Turtle Creek near Beloit, Wisconsin on a single field trip. About two hundred specimens were taken on the trip so that the seeming hermaphroditic ones constituted about 2.5 per cent. of the entire catch.

Diligent search was made in the same locality during the late spring with the result that three more similar specimens belonging to the same species were found.[1] The eight specimens are referred to in the descriptions as numbers 1 to 8 inclusive.

The search was continued in 1923 resulting in the capture of four females in the same locality in Turtle Creek. All showed

[1] Six of the eight specimens described have been deposited in the Carnegie Museum at Pittsburgh.

definite secondary sexual characters of the male. All four were carrying eggs or embryos attached to the abdomen, making it certain that they were fully functional females in spite of the presence of male secondary sexual characters. These four will be referred to in the descriptions as numbers 9, 10, 11 and 12. The brood of one of the specimens was successfully reared. Eight months after hatching twelve members of the brood were still thriving and it is hoped that the effort to maintain and breed them under laboratory conditions will be successful.

Later in the summer of 1923 one hundred females were taken from South Kinnikinick Creek a tributary of the Rock River fifteen miles south of Beloit. Among these were found seven having the partial development of male secondary sexual characters. These seven will be referred to as numbers 13, 14, 15, 16, 17, 18 and 19.

Other streams in the vicinity of Beloit have been searched and a considerable number of abnormalities in the secondary sexual characters have been found. In no female individual, however, has there been found any male secondary sexual character.

The fact that this simultaneous occurrence of male and female secondary sexual characters in the same individual has been found only thirteen times in the entire genus *Cambarus* makes the discovery of nineteen specimens in a single species within a limited area well worth investigating.

This same condition has been found to exist in southern Wisconsin to a still greater degree in *Cambarus virilis*. Eighty-eight per cent. of all the females taken in Delavan Lake show additional, male secondary sexual characters.

It seems likely that the conditions causing the high rate of peculiarities is a local one and it is proposed to go into the matter thoroughly and to publish results whenever a body of new data has been collected or whenever any conclusions have been reached.

Review of Literature.

The first described instance of a truly hermaphroditic decapod crustacean is that of a lobster (*Homaris vulgaris*) which had normal external genitalia and internal organs of the male on the left side and those of a female on the right side. The specimen

was described by F. Nicholls in 1730. Another specimen has been described in the Canadian Naturalist, Vol. XXXIII, No. 2.

Rosseau and Desmarest described several cases in *Astacus fluviatilis* of branched oviducts opening on the third and upon the fourth walking legs.

Von Martens described three specimens of *Cheraps preisii*, an Australian crayfish, in which there were openings at the base of the third walking legs but no oviducts and no ovaries. The specimens were normal males except for this peculiarity.

Von Ihring, Lönnberg, Faxon and Hay working upon various species of Parastacus, a genus found in South America, found that there were three constant conditions of the genital glands, ducts and external genitalia. Two of these conditions pointed toward a state of partial hermaphroditism. In some species there were single genital glands, either spermaries or ovaries, and two sets of genital ducts and genital openings. Only one of the sets of tubes was functional, however. In other species a single genital gland and two sets of genital tubes existed but there were no genital openings for the non-functional tubes. In still another species there was no evidence either of extra genital tubes or of extra genital openings. The normal condition in the first two subdivisions of this genus was, therefore, one of partial hermaphroditism.

Faxon (1885) found four cases in the genus *Cambarus* which gave some evidence of hermaphroditism.

One specimen of *Cambarus propinquus sanborni*, 60 mm. long, had the claws of a female, the abdominal appendages of a female and a well-formed annulus but no female genital openings at the base of the third walking legs. Instead, it had the external genital openings of a male at the base of the fifth walking legs. Dissection demonstrated the presence of a well-developed ovary so the specimen was evidently a female.

The second case was also one of *Cambarus propinquus sanborni*, 38 mm. long. The specimen was female in every respect except that the first abdominal appendages were like those of the male.

The third case was a specimen of *Cambarus diogenes*, 84 mm. long, in which the external genitalia were those of a female with the exception of the first abdominal appendages which were

almost as large as those of a male but were lacking the characteristic, recurved hooks.

The fourth specimen, a female of Cambarus *propinquus*, 72 mm. long, was normal with the exception of the first abdominal appendages. These were modified so as to be almost identical with but slightly smaller than those of the male.

Hay (1905) in examining the collection of crayfishes in the U. S. National Museum which had been made by the U. S. Fish Commission and by himself found four additional specimens which showed evidence of hermaphroditism.

In the first specimen, *Cambarus spinosus*, the organs internally and externally were those of a female with the exception of the annulus ventralis which was rather small. In addition there were the following male secondary sexual characters: hooks on the base of the third walking legs, abdominal appendages modified as in the male but slightly undersized, genital papilla at the base of the fifth walking legs, but no genital pores.

The second specimen, also *Cambarus spinosus*, was identical with the first with the exception of the first abdominal appendages which were shorter.

The third case, a specimen of *Cambarus propinquus*, 53 mm. long, displayed a preponderance of male secondary sexual characters on the left side and of female characters on the right side. The right side had the usual genital pore of the female at the base of the third walking leg, a small abnormal appendage on the first abdominal segment and the usual second abdominal appendage of the female. The annulus ventralis was also well developed. On the left side there was no female genital opening at the base of the third walking leg but on this third leg there was the copulatory hook normally found in the male. The first and second abdominal appendages of the left side were those of a normal male and at the base of the fifth walking leg there was the genital pore of the normal male. Nothing of the internal structure could be made out.

The fourth case appeared in a specimen of *Cambarus affinis*, 106 mm. long. Externally it had all the characters of a male except that it had no opening for the sperm duct at the base of the left fifth walking leg. It did have, however, the genital openings

of the female at the base of the third walking legs. A dissection revealed that there were fully developed ovaries and oviducts on both sides and a small testis with a sperm duct on the right side.

Hay concludes that in the genus *Cambarus* hermaphroditic individuals are females "which owing to some ambiguity of the formative cells in the embryo have developed to a greater or less degree the characters of the opposite sex. The condition is a very rare one and is usually shown in the external organs only". . . . "Hermaphroditism is as uncommon in *Astacus* as in *Cambarus*. Among the Parastacidæ the condition of apparent hermaphroditism seems to be established in the genus *Parastacus*. It may also be found in *Cambarus* but is rare or altogether wanting in other genera."

Ortman (1905) described five additional cases in the genus *Cambarus*, four collected by himself and one by E. B. Williamson.

His first case was one of *Cambarus obscurus* which had as female secondary sexual characters the female type of claw, a definite but small annulus and no hooks on the third walking legs. The specimen had, however, the genital openings of the male at the base of the fifth walking legs and the first and second abdominal appendages, clearly of the male type.

The second specimen was also one of *Cambarus obscurus* which had not only the annulus and the genital openings of the female but also copulatory hooks on the third leg (a male characteristic). The abdominal appendages were abnormal but full-sized and male-like. The specimen was interpreted as being a female with the full but not specific development of male organs.

In the third case the specimen (*Cambarus bartoni*) was a functional female, 71 mm. long with eggs attached to the abdomen. It showed the full equipment of secondary sexual characters of the female and in addition a pair of hooks at the base of the third walking legs.

The fourth individual, also a specimen of *Cambarus bartoni* (48 mm. long), had female claws, a well-developed annulus and the second abdominal appendages of the female. The usual hooks at the base of the third walking kegs were lacking. The male secondary sexual characters were shown in the genital openings at the base of the fifth walking legs and in the full development of the male first abdominal appendages.

The specimen collected by Williamson was one of *Cambarus rusticus*. The first abdominal appendages were not fully developed but were clearly of the male type while all the other secondary sexual characters were of the female type.

Description of New Cases.

Specimens 1, 2, 3, 4 and 5. (Fig. 5.) These five specimens are identical in their peculiarities and it will therefore suffice to describe one of them and to give the lengths of the other four. The length of the specimen described is 48 mm. The lengths of the others are 45, 47, 49 and 52 mm. respectively. In the specimen 48 mm. long the secondary sexual characters are typically female but there are the following additional characters: copulatory hooks are present at the base of the third walking legs; the first abdominal appendages are modified to resemble somewhat those of the male of the first form, being shorter, however, (8 mm. in length) with the tips undivided and curved toward the median line (Fig. 10); the second abdominal appendages are those of a normal male (Fig. 10). A dissection of the internal organs has been made and ovaries and oviducts have been found. The eggs are full sized and would normally have been laid within two months. The ovaries are heavily parasitized by the embryos of a flatworm [1] to such an extent that many of the eggs are entirely replaced by the encysted embryos. No trace of a testis or of a vas deferens is present.

Specimen number 6 is 42 mm. long. It differs from numbers 1, 2, 3, 4, and 5 only in the degree of development of the first abdominal appendages. The right member of the pair is in all respects like the homologous appendage of the normal male of the second form. The left member of the pair is slightly shorter and the tip is bent toward the median line.

Specimen number 7 is 54 mm. in length and is typically female in all its secondary sexual characters except in the case of the two abdominal appendages. The first abdominal appendages are modified like those of cases 1, 2, 3, 4 and 5 and are of the same size. The second abdominal appendages differ only slightly from those of the female, being a little stronger and the inner

[1] The writer is indebted to Professor A. S. Pearse of the University of Wisconsin for identifying the parasites as *Microphallus opacus* Ward.

ramus a little thicker. There is no trace of the triangular shoulder which occurs regularly on this appendage in the normal male. This specimen has been dissected and as in the previous cases there have been found well developed ova. This case is unquestionably one of a female with the partial development of male secondary sexual characters. The male characters are not so strongly developed as in the previous cases, the hooks on the third walking legs being absent and the second abdominal appendages being modified but little from the usual female type.

Specimen number 8 is 47 mm. long. It is without doubt a female, having a normal annulus ventralis and oviducal openings at the base of the third walking legs. The first abdominal appendages are very peculiar, however, being unlike on the two sides. The right one is a normal diminutive female appendage. The left one (Fig. 11) appears to have grown as a bifurcated female appendage but there has grown out of it at the junction of the basal and distal parts an appendage somewhat similar to and a little smaller than the normal first abdominal appendage of the second form male. The proximal half of the appendage is quite slender. The second abdominal appendages, both in size and general form resemble the normal female appendages but a distinct triangular shoulder like that found in the male is present upon the endopodite (Fig. 14).

Specimen number 9 was bearing well-developed eggs upon the abdomen when taken so there was no question as to the sex. It is 67 mm. in length. The oviducal openings and the annulus ventralis are those of a normal female but the following male structures are also present: copulatory hooks on the third walking legs; first abdominal appendages much like those of the normal first form male; second abdominal appendages like those of the male in form but a little smaller and with the triangular shoulder on the endopodite not so well developed (Fig. 4).

Specimen number 10, 55 mm. long, is unusual in that it possesses an extra oviducal pore at the base of the left fourth walking leg in addition to a rather complete complement of both male and female secondary sexual characters (Figs. 3, 9 and 14). The annulus ventralis and oviducal pores at the base of the third walking legs are normal female characters. An extra oviducal opening is present on the left fourth walking leg but it is

small and has no oviduct attached to it. The openings of the sperm ducts at the base of the fifth walking legs are in the same position as those of the normal male but are smaller and are not so prominent. The first abdominal appendages are shaped much like those of the normal, second form male but they are a little smaller and are more slender in the basal third. The second abdominal appendages are like those of the normal female with the addition of a marked triangular shoulder upon the endopodite, indicating a tendency on the part of the appendage to develope specific male characteristics.

This specimen was dissected and it was found that the internal structures were those of a female. The extra oviducal opening and the openings at the base of the fifth walking legs had no internal tubes connected with them. There was no trace of a spermary.

Specimen number 11 is 62 mm. long and is identical in its external peculiarities with specimen number 9. Like specimen number 9 also, it was bearing young embryos upon the swimmerets when taken and was of course a functional female. With the eggs so recently discharged it would be expected that the ovary would be depleted and small. A large genital gland has been found, however, which is irregular in shape and seems to be made up of unlike parts. It is possible that the structure may prove to be an ovo-testis.

Specimen number 12, 53 mm. long, when taken was bearing embryos which were in an advanced stage. These embryos were reared in the laboratory and forty-nine of the sixty reached a stage in which the secondary sexual characters had become distinguishable. All the embryos were female and all were normal. As in specimen number 11 a large irregular genital gland has been found but there are no ducts except the normal oviducts. Externally this specimen is with the exception of its size almost identical with specimen 11.

Specimens number 13 to 19 inclusive (Fig. 6) are alike in their peculiarities, all being normal females as evinced by their internal and external organs but bearing in addition one male secondary sexual character, namely a hook on the left third walking leg. The lengths of the specimens number 13 to 19 are respectively 63 mm., 47 mm., 49 mm., 47 mm., 42 mm., 45 mm., and 50mm.

Discussion.

The occurrence of both male and female secondary sexual characters in the same individual is so rare and sporadic that it has seemed futile to speculate as to its cause. However, when nineteen instances are found in a limited area within two years it would seem that a close study of conditions might yield results. Two possible explanations suggest themselves.

I. The gonads may have been damaged by parasites so that the possible control of the development of the secondary sexual characters would be lacking. Parasites [2] were found, indeed, in great numbers in both males and in females and there can be no doubt that they did extensive damage to the tissues of the crayfishes. Ovaries, testes, alimentary canals, green glands and livers were often heavily infested and partially destroyed. The seeming effect of parasitism of the gonads upon the development of the secondary sexual characters has been worked out in great detail by Smith (1910). This author found great numbers of the Spider Crab (*Inachus*) so parasitized by *Cirrepede Sacculina* that the ovaries and spermaries of *Inachus* were frequently destroyed. The findings with regard to the effect upon the secondary sexual characters and his conclusions may be found in the following statement: "Whereas the males under the influence of the parasite, were capable of assuming all the female secondary sexual characters and under certain conditions might even develope ova in their testes, the infected females on the other hand, although the ovaries in some case completely disappeared, never approached to the male primary or secondary sexual characters in the slightest degree." ". . . The presence of *Sacculina* caused young females under 13 mm. in carapace length to assume prematurely the adult type of abdomen and abdominal appendage." ". . . it would appear that the male was a potential hermaphrodite and the female purely female."

If the irregularities described in *Cambarus propinquus* are due to parasitism it might be expected that the number of irregularities would be somewhat proportional to the number of heavily parasitized individuals. This, however, does not prove to be the case for parasitism is quite common and irregular secondary sexual characters are rare. Again, if parasitism causes these

irregularities some principle other than that found by Smith for *Inachus* must be sought in *Cambarus propinquus* to explain the relations between the gonads and the secondary sexual characters for in *Inachus* "the male is potentially hermaphroditic and the female purely female" while in *Cambarus* in the instances cited the male shows no tendency to develope secondary sexual characters of the female although the female in all the cases described has developed more or less fully the secondary sexual characters of the male.

II. In *Parastacus* the presence of both male and female secondary sexual sharacters in the same individual is a fixed condition which is transmitted from one generation to another without change. The question arises as to whether or not the condition may not be transmitted in *Cambarus* as in *Parastacus* although much less firmly fixed in *Cambarus* than in *Parastacus*. The finding of a considerable number of specimens within a very limited region would tend to strengthen the view that the condition was somewhat constant in *Cambarus* and was being transmitted. The females seem to be unaffected by the presence of the male secondary sexual characters and they are certainly sexually functional as shown by the fact that all such specimens taken during the breeding season were bearing eggs or embryos. One of these broods was reared and about fifty out of the sixty embryos were brought to a stage in maturity where their sex could be determined. It is rather suprising that every one of those brought to maturity was a female. Twenty-one were carried without mishap to a stage when the secondary sexual characters were fully developed and all were normal, showing no traces of the conditions which existed in the mother.

It is noteworthy that specimens taken within a short distance of each other at the same time should closely resemble each other in their deviation from the normal. The specimens taken from Turtle Creek in 1922 show the peculiarity in the first abdominal appendages illustrated in figure 10. Those taken from Turtle Creek in 1923 possess first abdominal appendages like those illustrated in figures 9 and 11 while the seven taken from South Kinnikinick Creek are all alike in having a single male character, one hook on the left third walking leg. This would indicate that the individuals resembling each other might have been members of the same brood.

To say that the irregularities in the secondary sexual characters may be transmitted is not to answer the question of their origin. It would imply, however, that the cause producing the effect was deeply seated in the germ cells and was not brought about by a temporary, somatic influence.

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EXPLANATION OF PLATES.

PLATE I.

FIG. 1. Diagram showing the three basal segments of the five walking legs and the first two abdominal appendages with the second pair of abdominal appendages turned back. Normal male. I., II., III., IV., V., walking legs; *AP*1 and *AP*2, first and second abdominal appendages; *H*, copulatory hooks on third walking legs; *S*, external openings of sperm ducts at base of the fifth walking legs.

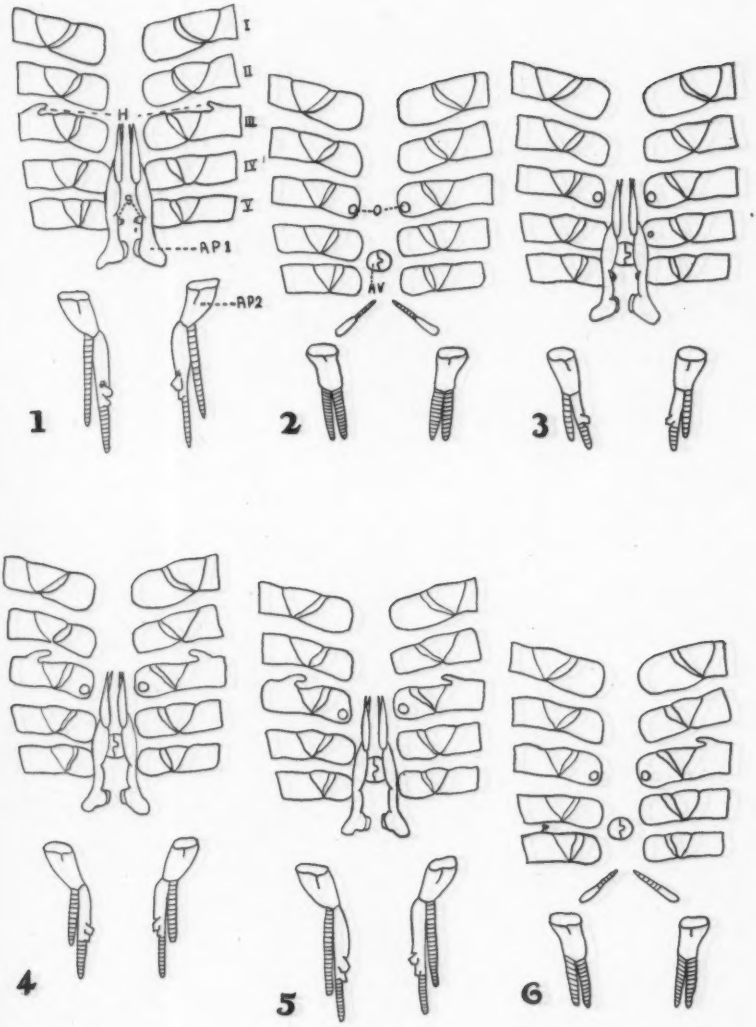
FIG. 2. Diagram illustrating the three basal segments of the five walking legs and the first two abdominal appendages with the second appendage turned back. Normal female. *O*, oviducal openings at the base of the third walking legs; *AV*, annulus ventralis. Other explanations as in Fig. 1.

FIG. 3. Diagram illustrating the three basal segments of the five walking legs, the first two abdominal appendages and the peculiar features described in specimen number 10. Structures may be identified by the explanations in Figs. 1 and 2.

FIG. 4. Diagram of three basal segments of walking legs and first two abdominal appendages with other features peculiar to specimen number 9.

FIG. 5. Diagram of basal segments of walking legs and first two abdominal appendages with other secondary sexual characters shown in specimens 1 to 5 inclusive.

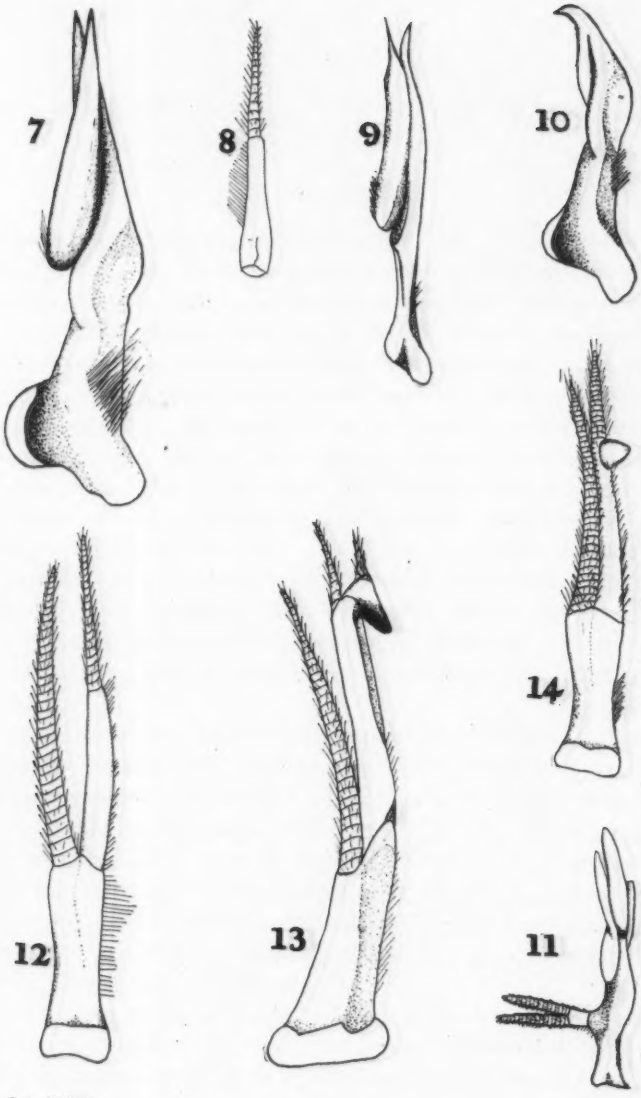
FIG. 6. Diagram to illustrate the condition of the secondary sexual characters in specimens 13 to 19 inclusive.



C. L. TURNER.

PLATE II.

FIG. 7. Left first abdominal appendage of normal male. × 7.5.
FIG. 8. Left first abdominal appendage of normal female. × 7.5.
FIG. 9. Left first abdominal appendage of specimen number 9. × 7.5.
FIG. 10. Left first abdominal appendage of specimens 1 to 5. × 7.5.
FIG. 11. Left first abdominal appendage of specimen number 8. × 7.5.
FIG. 12. Right second abdominal appendage of normal female. × 7.5.
FIG. 13. Right second abdominal appendage of normal male. × 7.5.
FIG. 14. Right second abdominal appendage of specimens number 8 and number 10. × 7.5.



C. L. TURNER.

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THE SURFACE TENSION THEORY OF MEMBRANE ELEVATION.

L. V. HEILBRUNN.

When a sea-urchin egg is inseminated it lifts off a membrane from its surface. A similar phenomenon can be produced by various reagents. Many explanations have been advanced to account for it. Some years ago, I showed that all substances which produce membrane elevation are those which might be expected to produce a lowered surface tension (1). Also, in general, substances which lower surface tension markedly, produce membrane elevation. And so I advanced the view that surface tension lowering was in all cases related to membrane elevation, and I offered a physical explanation of the process based on such a lowering of surface tension. The idea was new, although Traube (2) had previously shown that when substances of a chemical series are compared, those with lower surface tension are relatively more effective in producing membrane elevation. Traube thought the process involved a secretion on return to sea-water.

The surface tension theory of membrane elevation was accepted by some workers (3) but not by others. Garrey (4) in a review of the literature states that the theory demands the existence of a membrane on the uninseminated egg. For this Garrey finds no evidence, and he cites Moore's (5) repetition of Ziegler's (6) and the Hertwigs' (7) experiment with broken-up eggs as an argument against such a membrane. The evidence for a pre-existent membrane is abundantly supplied in my 1915 paper, to which Garrey does not refer.

In a paper published recently, Just (8) claims that hypertonic solutions of sodium chloride in sea-water, which do not lower surface tension, nevertheless produce membrane elevation.[1]

[1] As a matter of fact hypertonic solutions of NaCl in sea-water do secondarily produce lowered surface tension, for they cause membrane swelling and this produces lowered surface tension.

In my 1915 paper, I pointed out that the earlier descriptions of cortical change in the sea-urchin egg had failed to distinguish between two types of cortical change. The normal change at fertilization is a membrane elevation. Many reagents produce this change, others however produce a swelling of the membrane. The two types of change, although fundamentally quite different, are not easy to distinguish morphologically, partly because the surface of the egg is not especially favorable for microscopic observation. I therefore proposed various criteria to distinguish them. Thus elevated membranes collapse in albumen solutions, swollen membranes do not. Other criteria are easily established. When eggs with elevated membranes are crushed, they flatten and obliterate the perivitelline space. Eggs with swollen membranes have little or no perivitelline space, and when they are crushed, the thick membranes remain around them as before.

Just tried none of these criteria. The membranes he describes in his paper seemingly lack a perivitelline space, for he notes that on return to sea-water from the hypertonic solution, the perivitelline space is not obliterated. If the membranes were separated off as Just believes, and if there were a perivitelline space, one would think that this space would be obliterated by the osmotic expansion of the egg on return to ordinary sea-water.

As soon as an opportunity was afforded, I repeated Just's observations. With the concentrations he used (20–24 per cent. 2½ *M* NaCl in sea-water), no membrane elevation or separation could be observed. In such solutions the membrane could be seen slowly to expand and swell. There was no evidence at all of a sudden movement of the membrane such as occurs when the membrane is elevated. Tested with albumen solutions the membranes did not collapse. When the eggs were compressed the egg contents did not expand to the outer limits of the membrane. The membranes produced by solutions of sodium chloride in sea-water, or on return from such solutions to sea-water, were certainly swollen and not elevated. In the light of this evidence it appears that the argument advanced by Just is not valid. *It still remains true that all substances which produce typical membrane elevation do in every instance cause a lowering of surface tension.*

It should perhaps be pointed out that in cases of extreme coagulation of the protoplasm, the contents of the egg may sometimes be made to shrink away from the vitelline membrane. No good case of this is known for the sea-urchin egg, although some shrinkage apparently occurs after prolonged heat coagulation. In the *Cumingia* egg, which has a much stiffer membrane than the sea-urchin egg, a membrane which does not normally become elevated, some 1916 experiments showed that the protoplasm would shrink away from the vitelline membrane when the egg contents had been thoroughly coagulated after several hours exposure to hypertonic solutions. Such slow coagulative shrinkages are scarcely to be confused with true membrane elevation, although in the *Cumingia* egg under certain conditions the membrane may become stretched away from the egg after it is released.

Just makes one other point against the surface tension theory. He states that in "the eggs of *Arbacia* and *Echinarachnius* any competent observer can see that membrane separation following insemination is no mere surface tension effect." This is not a very serious argument. The surface tension theory claims only that the lowered surface tension is the cause which underlies the process, not that all the details which follow the initiation of the process are surface tension phenomena. And anyway how can one decide a physical problem by mere visual observation?

Just, apparently, has attempted to do this. The lifting off of the membrane is a physical process and demands a physical explanation. Just's nearest approach to this is his description of membrane elevation as involving a "wave of negativity" (9). But he does not use the word in any known physical sense.

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THE INDEPENDENT DIFFERENTIATION OF ISOLATED CHICK PRIMORDIA IN CHORIO-ALLANTOIC GRAFTS.

I. The Eye, Nasal Region, Otic Region, and Mesencephalon.

LEIGH HOADLEY,

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I. Introduction.

The study of the power of independent growth and differentiation of embryonic primordia (self-differentiation, Roux) has been pursued by various methods. The experiments on the development of isolated blastomeres, inaugurated by Roux's study of the frog's egg, have contributed greatly to our knowledge of the localization of potentialities in cleavage stages. More recently, investigation of growth and differentiation of embryonic tissues has been carried on by other methods. The classical experiments of Harrison (1907 *a*, 1910) on the embryonic nerve cells of amphibia in tissue culture, followed by numerous studies of other embryonic cells by Harrison (1912), Lewis, M. R. and W. H. (1911 *a*, *b*, etc.), and others, furnish good examples of the specificity and the degree of the independent power of differentiation of the isolated cells of various embryonic organs. Harrison (1907 *b*) and others have also made studies of the independent power of growth and differentiation of limb primordia and other parts of the amphibian larva by a method having many additional advantages, that of grafting. The part studied was

grafted to another larva of approximately the same age. In such experiments, the grafted parts continue to develop while the graft is nourished and relieved of its excretions by the host. The differentiation of the organs of the host, which are developing simultaneously with those of the transplanted part, must, however, affect the differentiation of the transplant to a greater or lesser degree.

The ideal conditions for a transplantation in which it is desired to determine the independent power of growth and differentiation of the part, would be in a location where there are no nerves from the host, where there is no differentiation of host tissue to influence the grafted part, and where there is a sufficient supply of blood vessels to insure nutrition and the ability to excrete waste products on the part of the transplant. This must be in a rapidly growing tissue which is capable of repair, and such as will react to foreign tissue with rapid incorporation and a host of capillaries. The chorio-allantoic membrane of the chick furnishes such conditions as has been pointed out by Murphy (1913), Danchakoff (1916), Kiyono (1917), Minoura (1921), and Atterbury (1923).

It is my intention to attack the problems of independent growth and differentiation by the isolation and transplantation of the various primordia of the chick embryo to the chorio-allantoic membrane. A general survey of the field has already been made with a variety of tissues and has yielded promising results. For instance, when a cross section of the body of the thirty-six or forty-eight hour chick is transplanted, the grafts obtained show that cartilage, bone, muscle, nervous tissue, and mesonephros grow well. In one case of transplantation of the primitive streak, nephrogenous tissue is found to be present. In many cases of such grafts of cross sections of the body, small buds of the feather germs form and grow well; they receive a rich vascular supply. The present paper is confined to the organs of special sense, *i.e.*, the eye, nasal region, otic region, and one example of brain tissue, the mesencephalon. The mesencephalon was selected because of its close association with the eye, and because of its high degree of differentiation in the birds. Other parts will be considered in later papers.

The progamme was suggested by Prof. F. R. Lillie of this laboratory to whom I wish to express my appreciation for many helpful suggestions and criticisms during the progress of the work.

II. Methods.

In the experiments recorded in this contribution, I have used chicks of twenty-four, thirty-six, and forty-eight hours of incubation as the source of material. The methods of grafting employed are similar to those used by Murphy and Danchakoff with certain modifications developed at this laboratory. An egg of nine to ten days of incubation is candled and the juncture of two of the larger blood vessels is marked. The shell is then sterilized and a small window is cut through it at this point with a hacksaw blade. The egg is then returned to the incubator while the embryo which is to furnish the material for the transplantation is put in physiological salt solution on a warm stage under a binocular dissection microscope. For illumination, the light of a 150-watt, nitrogen-filled tungsten lamp is concentrated on the embryo by a bull's-eye condenser. All dishes are sterilized in the autoclave and all instruments are boiled in water. Dissecting needles which have been ground into fine knives are used in the operations. With these, the primordium is isolated and is then placed on the chorio-allantoic membrane as near as possible to the juncture of the two vessels noted above. The shell membrane which is torn slightly to admit the tissue is replaced, the shell is returned and the cut edges are sealed with paraffine. The egg is then returned to the incubator in such a position that the window is down, so that the weight of the egg insures the contact of the membrane and the implanted tissue. After twelve hours the egg is rotated, and at regular intervals thereafter rolled as any developing egg. Many of the transplants are sure to have more or less mechanical injury, and the time required for their incorporation within the membrane of the host and subsequent infiltration with blood vessels must vary. These are the most important variables with which the method must cope. After six or seven days the egg is opened and the development of the grafted tissue is studied macro- and microscopically.

III. Experiments.

A. The Eye.

A series of grafts of the optic primordium of the chick have been made by this method. The material was taken from chick embryos of twenty-four, thirty-six, forty-eight, and in a few cases sixty hours of incubation. In some cases the entire primordium was excised, in others only a part of it was used. There are various difficulties to be encountered in such an operation. The mesenchyme, at these early stages, is quite sticky, and considerable care must be exercised to remove the greater part of it from the vesicle. Particular care is needed if it is desired that no part of the brain be included in the transplants. At the twenty-four hour stage, the neural folds are open in the optic region and only a slight swelling indicates the position of the optic foveola. The anterior end of the head was removed from such embryos; the region from which the optic vesicles develop was then bisected, and the primordium of each side was transplanted separately. The lens ectoderm is not yet definable so that transplantation of this region with that of the future optic vesicle is a matter of chance. At the thirty-six hour stage, the technic is much more simple. Here the embryo has from twelve to sixteen somites. Some of the embryos of this age were more advanced, but the majority fall within these limits. The primordium of the eye is then in the vesicle stage and is easily removed. Great care must be taken that the tissue is not harmed in the transplantation.

Another set of experiments was made on the eye at the thirty-six hour stage. The main part of the experiment was the same, but the lens ectoderm was dissected away. This is very difficult to do, for there is a great tendency for the wall of the optic vesicle to tear. In most of the cases which have been operated in this way, no lens ectoderm was left on the vesicle, but one case which later developed small portions of lens tissue is in doubt. The mechanical injury in an operation, must, of necessity, be great, and this is probably the cause of the greater degree of disorganization found in these grafts. Grafts of the isolated lens ectoderm were also made but no growth was found in any case, possibly owing to the very small size of the piece of the embryo transplanted.

With the experiments made from the eye primordium of the forty-eight-hour embryo, greater difficulties with the elimination of the surrounding tissue began to appear. It is practically impossible to isolate absolutely the optic cup of this stage from the surrounding mesenchyme without injuring the cup in some way. The optic cup can be dissected out with little difficulty, but in every case there may be seen a fur-like border of the mesenchymal cells attached to its surface. After a few attempts to remove this, it was considered to be useless, for the manipulation injured the cup in every case. On this account, when a transplantation of the optic cup is spoken of, it means that the optic cup together with the immediate mesenchyme was taken. Attempts were made here, as in the experiments with the thirty-six hour embryos, to remove the lens primordium from the optic cup. It was soon found that, though this is possible, there is a great deal of mechanical injury involved. Such experiments were therefore omitted. Since the sixty-hour "eye" is in much the same condition, the same difficulties were experienced with it, but it was not considered necessary to perform many experiments with the material from this age, for even at thirty-six hours of incubation, the cells of the optic primordium possess complete power of independent self-differentiation.

In the grafts obtained from the foveola of the twenty-four hour embryo, there is much variability in growth and differentiation. This seems to be due to the fact that there is a certain amount of crushing in the removal of the tissue, the disorganization produced by this mechanical factor increasing as the size of the operated part deceases. In one of the grafts there is a primary differentiation into tapetal cells which contain pigment granules, and retina-forming cells. Many of the grafts show nervous tissue but it is not sufficiently differentiated to warrant any positive statement as to its nature. There is much other tissue transplanted with the optic primordium of this age. In one of the grafts well defined ganglion cells are present. In no case, however, does any eye graft of this age show more than the primary differentiation into pigmented tapetal cells and unpigmented retina-forming cells.

The grafts of the optic vesicle plus the lens ectoderm of the

thirty-six hour embryo will be considered next. At this stage the optic primordium possesses great power of independent growth and differentiation. Many grafts were obtained and sectioned, a number of the most successful of which will be discussed here.

FIG. 1. Graft of an eye from a forty-eight hour chick embryo in alcohol before section, 19 *E* 13. (× 10.) For explanation, see text, page 290.

All of the cases which show any growth at all, prove, on microscopical examination, to have a primary differentiation of the tissues into a pigmented tapetal portion, a non-pigmented retinal portion, and a group of lens-forming cells. The results vary all the way from grafts in which there is no great differentiation of the retina and lens, to the more successful ones in which the retina is differentiated and the whole transplant appears approximately normal.

The more successful grafts show a high degree of differentiation though great variation in the extent to which the subordinate parts of the organ have differentiated. It is an interesting fact that the basal layer of the retina often shows many cells in active mitosis, *i.e.*, the graft is still in active growth. In the best graft obtained (Fig. 2), there is a very clearly defined retina with the layers developed sufficiently to warrant the statement that it is as far advanced as the normal; the lens is perfect, showing the anterior epithelial layer and the lens nucleus; the anterior and posterior chambers are present; the tapetum and the cartilage

of the eye are typical of the normal of the same age. The ciliary processes show around the lens. This case, 9 *A* 1, is therefore selected for detailed description.

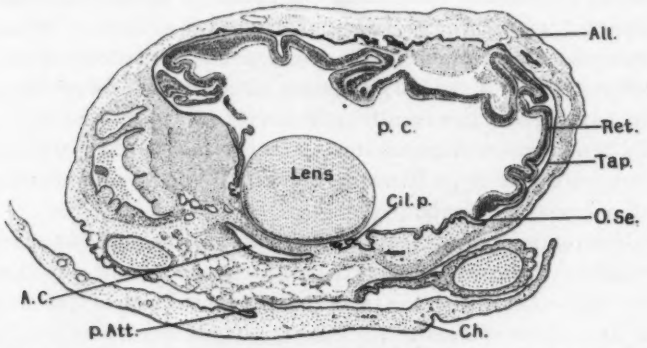

FIG. 2. Cross section of graft of eye grown from thirty-six hour primordium. Total age, nine and one-half days, 9 *A* 1. (× 20.) *A.C.*, anterior chamber. *All.*, allantoic portion of investing membrane. *Ch.*, chorionic portion of membrane. *Cil.p.*, ciliary process. *O.Se.*, ora serrata. *P.Att.*, point of attachment of the graft to the main portion of the membrane. *P.C.*, posterior chamber. *Ret.*, retina. *Tap.*, tapetum.

On March 24, 1922, the optic vesicle of a thirty-six hour chick was removed as free from adjacent parts as possible and was placed near the juncture of two of the largest blood vessels on the chorio-allantoic membrane of a chick of nine and one-half days' incubation. The egg was then returned to the incubator where development proceeded until April fourth when the egg was opened and the graft, which was large, removed. In my notes, taken at the time, I notice the remark that the graft was heavily pigmented.

Upon microscopical examination of the sections, the degree of differentiation of the graft proved to be quite remarkable. In only a few places is mitosis in the basal retinal layer common, *i.e.*, at places where there are large folds protruding into the posterior chamber. By far the greater part of the tissue has ceased proliferating and is differentiating. It may be well to consider this differentiation under four headings; the chambers, the tapetum and retina, the lenticular region, and the surrounding tissue.

The chambers in this case are well developed. A definite anterior and posterior chamber are present separated by the lens, the lenticular zone, and a thin layer of mesenchymatous tissue which is covered with an endothelium. The entire anterior chamber is lined by this tissue. The posterior chamber is large and is lined throughout by the retina save at the anterior border, where the lens and ciliary processes occur, as mentioned above. It is not regular, however, for at intervals, as can be seen (Fig. 2), the retina has proliferated to such an extent that there are folds entering the cavity. In no place do these obliterate the chamber as in some of the grafts.

The retina and the tapetum are well formed, though where convolutions of the retina occur, the tapetum does not always follow. On close examination, the tapetum appears to be formed of three or four layers of heavily pigmented cells arranged very closely. All of the layers of the retina are present (Fig. 3) but the layer of

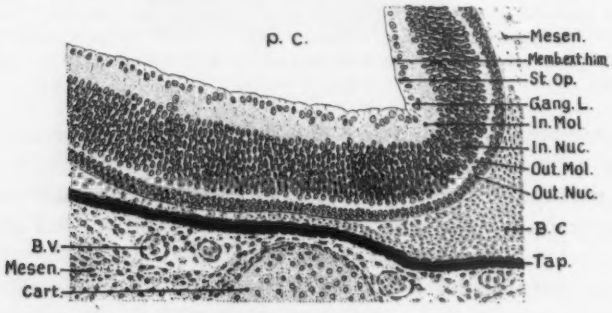

FIG. 3. Cross section of retina from same graft as Fig. 2. (× 180.) *B.C.*, blood corpuscles. *B.V.*, blood vessels. *Cart.*, cartilage. *Gang.L.*, ganglionic layer. *In.Mol.*, inner molecular layer. *In.Nuc.*, inner nuclear layer. *Memb. Ext.Lim.*, external membrana limitans. *Mesen.*, mesenchyme. *Out.Mol.*, outer molecular layer. *Out.Nuc.*, outer nuclear layer. *St.Op.*, stratum opticum. Other abbreviations as in Fig. 2.

rods and cones shows but little differentiation, a condition corresponding to that found in normal eyes of chicks of the same age. Bordering the cavity is the very thin membrana limitans interna. This separates the cavity of the eye from the stratum opticum which lies just beneath it. This is clear and easily distinguished from the sparsely nucleated outer ganglionic layer. Between this

and the inner nuclear layer is an inner molecular layer, very granular in appearance and containing only a very few scattered nuclei which have failed to associate as yet with either of the adjoining layers. The inner nuclear layer is nearly three times as wide as the molecular layer and is very heavily nucleated. Separating this from the outer nuclear layer is the outer molecular layer, also free of nuclei. As mentioned above, the rods and cones have not differentiated at this stage. At places, especially in the region of the retinal folds, there are connective tissue cells which fill in the spaces between these folds and the tapetum.

The differentiation occurring in the lenticular region is quite striking (Fig. 4). The tapetum continues anteriorly to a point

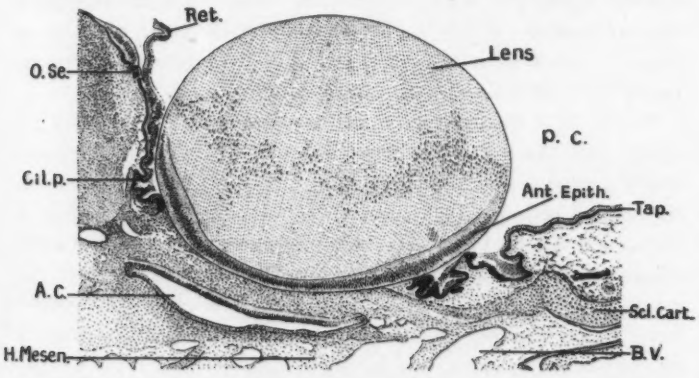

FIG. 4. Lens and lenticular zone of same graft as Figs. 2 and 3. (× 50.) *Ant.Epith.*, anterior epithelium of lens. *H.Mesen.*, host mesenchyme. Other abbreviations as in Figs. 2 and 3.

just mediad to the margin of the lens where it doubles on itself in a pigmentless layer of cells. The ora serrata, ciliary processes, and the iris are present. The lens itself is supported by the suspensory ligament.

In its differentiation, the lens is quite typical (Fig. 4). It is oval in shape, being composed of a wide nucleus of fibers with their medially located nuclei, and an anterior epithelial layer. Both are identical with the same parts of the normal of the same age. The lens surface is toward the ectodermal portion of the chorio-allantoic membrane. The incorporation of the graft

within this membrane was so complete that no injury was done to these parts when the membrane containing the graft was removed from the shell of the host.

Very little tissue save those already mentioned occurs in this graft. The sclerotic coat of the eye is present throughout. To one side there is a small mass of brain tissue containing typical nerve cells and processes. This is heavily infiltrated with blood vessels and not easily identified. Adjacent to this region, the tapetum and retina are very much convoluted and disorganized, and a heavy infiltration of blood corpuscles among the folds makes the identification of definite structures impossible. The optic nerve and pecten which would be located in this area under normal conditions cannot be found. The disorganization and the infiltration of the blood cells may mask their position as degenerative changes are apparent. The entire graft is surrounded by the mesenchymatous tissue of the host.

In not all of the grafts is brain tissue present. A graft of the optic region of a thirty-five hour chick, case 14 *A* 1, when sectioned shows a very evenly shaped posterior chamber lined by well differentiated retina. This eye shows the same differentiation of retina and tapetum as graft 9 *A* 1 above, though there is a total absence of brain and cartilage tissue. The various layers of the retina are well defined. The cut edges of the excised optic vesicle have drawn together and the point at which they have fused is marked by a thickening and by slight convolutions of both tapetum and retina. No lens tissue is present in the sections, probably due to the failure of its primordium to be incorporated. The grafted tissue is surrounded by an extremely thin layer of host mesenchyme. In no place is there any sign of brain tissue, optic nerve, or pecten, though the point at which they would normally occur is definable.

Graft 19 *E* 13 from a forty-eight hour chick embryo, is somewhat similar to 9 *A* 1 above. This case, after seven days of growth, a total of nine days, showed a graft 4 mm. x 4 mm. in size. A drawing of this graft as it appeared in alcohol before sectioning is shown in Fig. 1. The heavy pigmentation of the greater portion of the surface will be noted, together with the absence of pigment from a portion of the upper face. It was

thought on macroscopic examination, that this would prove to be the lens, but on section, a very different picture presented itself. The unpigmented portion is the point at which the optic nerve leaves the retinal area and runs out into the mesenchyme (Fig. 5).

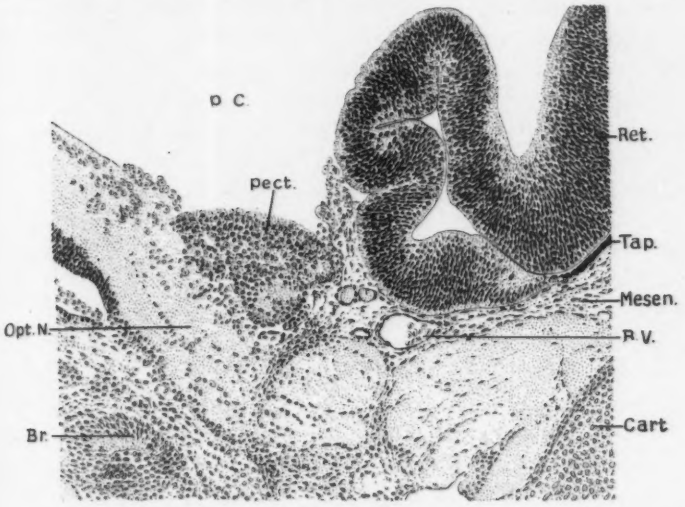

FIG. 5. Cross section of origin of optic nerve in graft, 19 *E* 13. Same as Fig. 1. (× 140.) *Br.*, brain. *Cart.*, cartilage. *Opt.N.*, optic nerve. *Pect.*, pecten. Other abbreviations as above.

The pecten is present though irregular in shape, due no doubt to the mechanical stress in the growth after transplantation. From this point the optic nerve grows out between the tapetum and the ectodermal wall of the chorionic membrane for quite a distance. The other white area in the graft is brain tissue which was transplanted with the optic cup. Only in the region of the nerve and nervous tissue is there any mesenchyme or cartilage in abundance. In the rest of the graft, the tapetum comes up close to the border of the chorionic or the allantoic mesenchyme of the membrane. The layers of the retina are all well differentiated. The ora serrata appears near the margin of a much distorted lens, ending on its lenticular border in a much convoluted and disorganized ciliary process. The misshapen appearance of the lens is apparently due to the fact that it lies next to the shell membrane and

is not completely incorporated by the chorionic overgrowth. At both ends of the sectioned material, where the stratum opticum is cut nearly transversely, the nerve fibers of this layer show very well. The whole graft compares very favorably with the normal eye of the same age.

In the experiments performed on the forty-eight hour chick, as in those cited for the thirty-six hour material, the degree of regulation seems to be dependent on the amount of injury to the engrafted tissue, both during manipulation and during the growth period which follows. My data show many cases where a much convoluted retina and tapetum are present with no posterior chamber showing. All degrees of lens malformation may occur. The retinal cells are always definable as such, though the degree of differentiation of the retina as expressed by zone formation varies considerably.

Five successful grafts were obtained from thirty-six hour primordia from which the lens ectoderm had been dissected away. The data on these cases are to be found in table I. In only one of

TABLE I.

GROWTH OF THIRTY-SIX HOUR OPTIC VESICLES WITH THE LENS ECTODERM REMOVED.

Case.	Control Age.	Remarks.
15 *A* 1	8½ days	Posterior chamber, retina and tapetum present. No lens tissue.
15 *B* 1	8½ days	Nervous tissue, cartilage, much convoluted retina, tapetum, small amount of lentoid tissue.
15 *C* 1	8½ days	Very little area of much convoluted retina and tapetum only.
16 *B* 1	8 days	Nervous tissue, retina and tapetum. No lens tissue.
16 *B* 2	8 days	Cartilage, retina, much convoluted tapetum. No lens tissue.

these grafts are any lens cells present. It is more reasonable to suppose that these are the result of the proliferation of some cells of the lens ectoderm which were not removed in the operation, than that they arose by a redifferentiation of the cells of the retina or iris (see Fischel for amphibian larvæ, 1900) or by a stimulation of the chorionic ectoderm by the optic cup (see Lewis 1907 and Spemann 1912, and other papers on the amphibian eye).

Spemann (1912), Lewis (1907 *a*, *b*, *c*,), and other workers have

found that the optic cup of the amphibian embryo has great power of self-differentiation when transplanted to other parts of the same or another embryo, while the lens ectoderm is dependent on contact with the optic cup for its development. The specificity of the ectoderm which forms the lens varies with the species employed. Lewis (1907 *c*) notes that the various parts of the cup develop independently "in marked contrast" to the lens which will not form unless the optic cup is present. If brain also is present, there may or may not be an optic nerve developed. The tissues seem to continue their own specific type of differentiation independent of one another.

The fact that in case 14 *A* 1 where no brain tissue is present, the optic nerve is also lacking, while in case 19 *E* 13 both are present, seems, therefore, to deserve more than passing notice. In the first mentioned case, the point of fusion of the cut edges of the primordium is definable. There is no indication of the optic nerve anywhere in this region. In case 9 *A* 1, while there is a small amount of brain tissue present, the position of the optic nerve cannot be found. It may be obliterated by the great number of degenerate convolutions in the retina and tapetum adjoining this region. In case 19 *E* 13, however, both optic nerve and pecten are very definitely located. The position of the optic nerve is the same as that of the optic stalk which connects the optic cup with the brain in the early stages of development. When this is removed, the optic nerve fails to develop. The fibers of the cells, instead of finding exit by this path, are retained in the stratum opticum. It would seem that these structures, as well as the parts of the retina and lenticular region arise from very definite locations in the primordium.

The experiments show very definitely that the various parts of the eye primordium have the power of independent self-differentiation to quite a remarkable extent. This conclusion is strengthened by the findings of Lewis, Spemann and others for the amphibian eye. The regulation of the parts to form a more or less perfect structure seems, on the other hand, to be dependent on various mechanical factors involved in the experiment.

B. The Nasal Region.

The material for the grafts of the nasal region was taken from chicks of thirty-six, forty-eight, and sixty hours of incubation. As in the case of the eye tissues, only a few grafts were made of the sixty hour material. Inasmuch as it was desired to note the growth and differentiation of the nasal epithelium especially, no twenty-four hour material was grafted, for at this age, the medullary folds only are localized.

When grafts were made of thirty-six hour chick primordia, the anterior end of the head as far back as the optic vesicles was transplanted. If the material was taken from an embryo of forty-eight hours incubation, the tip of the head was cut away so that both nasal pits were transplanted in one piece.

A brief account of the normal development of the olfactory organ based on Disse's account (1897) will form a useful introduction to the study of the grafts of this region to be described. After three days of incubation, the sensory epithelium of the chick nose is represented by two thickenings of the epithelium in the nasal grooves. As the embryo approaches the fourth day, the epithelium becomes differentiated into columnar epithelial cells and round cells, more transparent in sections stained with carmine. These cells are isolated in groups which are more or less distinct from each other. It is from these that the cells migrate out, forming the olfactory nerve and the ganglion-like group. At first a chain of cells appears to lead from the nasal epithelium to the brain. As late as the sixth day, this nerve primordium contains cell bodies but they are most numerous at the extremities. In the eight day chick, the stage which serves as the control for the grafts to be described below, Disse finds the nerve formed and two types of cells appearing in it at rare intervals. There is a pear-shaped unipolar cell, and a bipolar cell that is spindle-shaped. The greater part of the nerve is composed of nerve fibers. The cells are most numerous at the ends of this fiber bundle. At the distal end, near the nasal epithelium, the grouping of these cells and their shape led Beard (1885) to describe a ganglion-like mass which Disse describes merely as a group of cell bodies. Disse (confirming His, 1889) concludes that the nervus olfactorius arises from certain cells of the nasal epithelium and adds that it lacks a ganglionic portion.

The cartilages of the nasal capsule of the chick have been described by Cohn (1903). The prenasal cartilages and the nasal septum are present on the eighth day. The primordia of the three turbinals are formed. These are the only cartilages which arise from the mesenchyme transplanted in the grafts.

Graft 41 *B* 1 is a growth of the nasal region of a chick of thirty-six hours of incubation. The graft was removed after six days of growth on the chorio-allantoic membrane of the host, a total of seven and one-half days, and was 3 mm. x 3 mm. in size. On section, the various parts of the nasal region are found to be present. They compare favorably in their development with the normal region in the embryo of eight days of incubation.

The grafted tissues with the exception of a portion of the ectoderm are well surrounded by the mesenchymatous tissue of the host membrane. In it, four gross areas are definable, the nasal sacs, the nasal cartilages, the forebrain, and the epidermis of the head. With the exception of the epidermis, these have maintained approximately their normal relationships. The sections cut the region transversely so that at one end the nasal sacs appear, separated by the cartilage of the nasal septum. As the sections are traced posteriorly, the sacs become surrounded by a cartilaginous sheath, the prenasal cartilages. As the nasal sacs disappear posteriorly, the forebrain appears. It is heavily infiltrated with blood corpuscles which do not seem to be located in any organized vessels. Between these structures themselves, and separating them from the chorionic and allantoic borders of the host membrane is a good growth of mesenchyme. A more detailed description of these parts follows.

The nasal sacs are two in number and distinct (Fig. 6). They end blindly at both ends and are completely surrounded by mesenchyme, save at one place where the left sac retains its connection anteriorly with the epidermis of the head. Posteriorly they widen out forming cavities of some size. The epithelium which lines the cavities is columnar in type. Mitosis is fairly common in the entire region but is more commonly found near the cephalic border where the epithelium is noticeably thickened. For the most part this layer is sharply defined from the surrounding mesenchyme, so that it appears as if a membrane were present

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The grafted tissues with the exception of a portion of the ectoderm are well surrounded by the mesenchymatous tissue of the host membrane. In it, four gross areas are definable, the nasal sacs, the nasal cartilages, the forebrain, and the epidermis of the head. With the exception of the epidermis, these have maintained approximately their normal relationships. The sections cut the region transversely so that at one end the nasal sacs appear, separated by the cartilage of the nasal septum. As the sections are traced posteriorly, the sacs become surrounded by a cartilaginous sheath, the prenasal cartilages. As the nasal sacs disappear posteriorly, the forebrain appears. It is heavily infiltrated with blood corpuscles which do not seem to be located in any organized vessels. Between these structures themselves, and separating them from the chorionic and allantoic borders of the host membrane is a good growth of mesenchyme. A more detailed description of these parts follows.

The nasal sacs are two in number and distinct (Fig. 6). They end blindly at both ends and are completely surrounded by mesenchyme, save at one place where the left sac retains its connection anteriorly with the epidermis of the head. Posteriorly they widen out forming cavities of some size. The epithelium which lines the cavities is columnar in type. Mitosis is fairly common in the entire region but is more commonly found near the cephalic border where the epithelium is noticeably thickened. For the most part this layer is sharply defined from the surrounding mesenchyme, so that it appears as if a membrane were present

surrounding it. In the thickened areas, however, the boundary becomes indistinct, and in many places, cells may be seen which appear to be migrating from the epithelial layer into the mesenchyme. These areas represent the sensory epithelium and from them the olfactory nerve arises. A short distance from the nasal

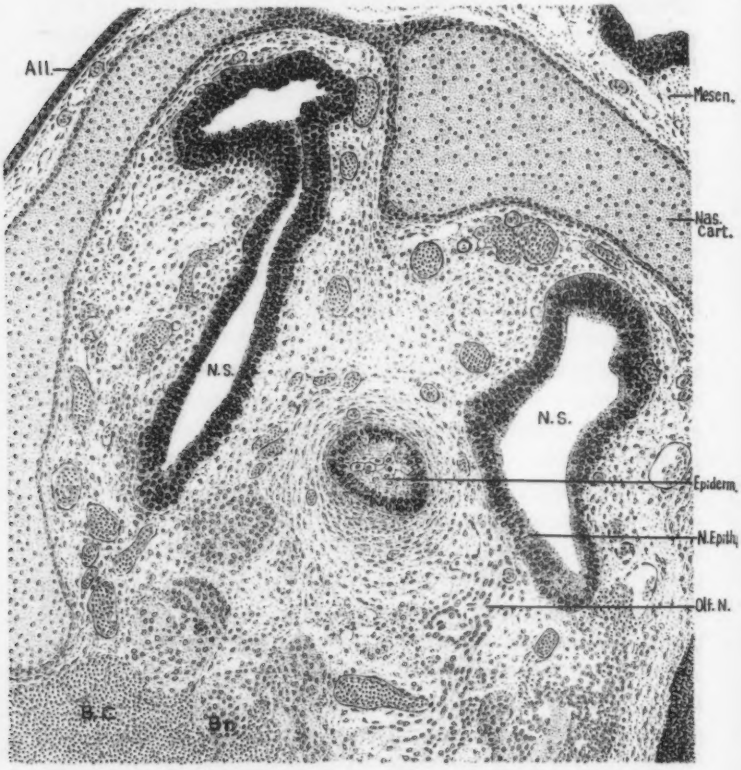

FIG. 6. Cross section of graft of nasal region of chick embryo of thirty-six hours of incubation, 41 *B* 1. (× 140.) *All.*, allantoic border of membrane surrounding graft. *B.C.*, blood corpuscles. *Br.*, brain. *Epderm.*, epidermis. *Mesen.*, host mesenchyme. *N.Epith.*, nasal epithelium. *N.S.*, nasal sacs. *Nas.Cart.*, prenasal cartilage. *Olf.N.*, olfactory nerve.

epithelium may be seen a small group of ganglion-like cells (Marshall, 1879) and toward these the nerve cells appear to be migrating. From the ganglionic group, nerve fiber bundles con-

taining a few cell bodies which correspond to the pear-shaped and spindle-shaped nerve cells of Disse, run to the forebrain where their course is lost in the disorganization present there. The large round primordial nerve cells of the nasal epithelium occur in the fiber bundles at rare intervals. The development of the nerve corresponds to that of the embryo of seven days of incubation as described by Disse.

The forebrain of the graft has no limiting membrane. The character of the cells is undoubtably that of brain cells but they are unorganized. This appears to be due to the fact that this tissue is so extensively infiltrated with blood vessels. In places there are what seem to be large sinuses filled with blood cells. Numerous small nerve-like masses are found in this region which are formed by union of the groups of the nerve processes. They end bluntly and blindly after penetrating the mesenchyme for short distances, resembling similar structures found in grafts of the mesencephalon which will be discussed below.

The cartilage which surrounds the nasal sacs has been mentioned above. This cartilage is in a single large piece but at intervals in the sections, there are breaks in its continuity. The nasal septum is present as a T-shaped structure, the top of the T forming a part of the floor of the region. Forming a roof over the nasal sacs is a crescent-shaped prenasal cartilage. This is broken at a single place on one side to permit the exit of a blood vessel. The cartilage does not persist through the entire graft but disappears shortly beyond the posterior end of the nasal sacs.

The epidermis of the head is present in the anterior end of the graft as an extensive, more or less cornified mass of tissue imbedded in mesenchyme. As it is traced posteriorly it approaches and finally, at the posterior level of the olfactory sacs, reaches the chorionic surface of the membrane where it is unincorporated by the host. A single tube-like growth of this epidermis has come to lie between the tip of the left olfactory sac and the forebrain.

As far as I have been able to discover, the only data on the differentiation of the nasal epithelium when isolated either completely or together with the immediate region, are furnished by Lewis (1907 *d*). This author records two experiments which show in the first place that the epithelium develops independently

of the forebrain, and secondly, that the nasal pit is capable of independent differentiation. Both sets of experiments were done with the larva of *Amblystoma*. In the first type of experiment, though the forebrain primordium was removed, the nasal epithelium sent out nerve processes into the adjacent mesenchyme, developing normally. The second type of experiment involve the transplantation of the excised nasal pits to a position beneath the ectoderm dorsal to the eye. Lewis (page 464) states that ". . . differentiation of the organ continued and nerve fibers were sent off into the region between it and the ectoderm, . . . but extend only a short distance."

In the grafts obtained from the transplantation of the nasal region of the chick embryo, the different parts of the organ show a high degree of specificity in the course of their differentiation. In comparing them with the control and with the description of the process of nerve formation by Disse, it was found that they show a condition comparable to growth in a normal embryo of the same age. The cartilage which is present, is, without a doubt, that of the transplanted part, and not of the host. The nasal sacs are present in nearly their normal relationships to the rest of the parts and have a differentiation closely resembling that of the same parts in the normal organ. The nasal epithelium gives rise to the nerve which is also present. The nerve cells of the forebrain differentiate nerve processes, the abnormal appearance in the sections being due to excessive vascularization and the absence of the limiting membrane, both of which are mechanical factors controlling the limits of differentiation to a great degree.

C. The Otic Region.

Grafts of the otic region and the otic vesicle are much more difficult to make than those of the nasal region. The auditory pit appears about the thirty-six hour stage, and the vesicle is closed about the forty-eight hour stage; the material grafted was taken from embryos of the last mentioned age in most cases. The first experiments were made by shelling out the otocyst and transplanting it, but it appeared very soon that the otocyst alone does not persist well in grafts. The mechanical injury due to the pressure of the egg itself, together with the adhesive quality of

the shell membrane due to its absorptive nature, resulted in no incorporation in the membrane of the host, and consequently no growth. I then tried transplanting the otic vesicle with the immediately adjacent mesenchyme, a slightly larger piece of tissue, and found that in such operations, though incorporation may not be complete and the mechanical injury more or less extensive, nevertheless, growth ensued. The investing tissue transplanted at the forty-eight hour stage includes only mesenchyme and somatic ectoderm. In all the operations, the brain wall was removed from the transplant before it was placed on the membrane of host.

Table II. is a record of the successful growths of the auditory

TABLE II.

DATA ON GRAFTS OF THE OTIC REGION.

Case.	Donor Age.	Control Age.	Remarks.
24 *D* 7	36 hr., 20 Som.	9½ days	Cartilage in crescent around epithelium of otic vesicle; epithelium differentiated. Incomplete incorporation.
25 *C* 5	48 hr.	9½ days	Cartilage independent of differentiated epithelium. Incomplete incorporation.
32 *A* 5	52 hr.	9¼ days	Differentiated epithelium in two parts, one surrounded by cartilage. Incomplete incorporation.
32 *B* 5	52 hr.	9¼ days	Cartilage near though not surrounding incompletely incorporated, differentiated epithelium.
37 *C* 5	48 hr.	8 days	Cartilage near epithelium. Small graft, epithelium differentiated.
38 *A* 5	48 hr.	8 days	Only small amount of unincorporated epithelium which is differentiated.
40 *B* 6	48 hr.	9 days	Cartilage and epithelium alone present. Epithelium differentiated.

vesicle. It will be noted that one of these is from a donor of thirty-six hours incubation, but the embryo showed a more advanced stage of development than is usual for chicks of that age. It corresponds more nearly to the forty-four-hour stage and consequently approaches the usual donor age, forty-eight hours.

Two criteria are employed in the identification of the parts of growths obtained in grafts of the otocyst. The morphological arrangement of the various structures aids greatly, but owing to the disorganization present in every case, this is supplemented by

close histological examination. In normal development, the cartilaginous otic capsule surrounds the sacculus, utriculus, and their derivatives, while the ductus and the saccus endolymphaticus are free in the mesenchyme. The histological differentiation of the several parts of the membranous labyrinth in the grafts is essentially the same as that of the control so that identification can be made positively by histological structure; this is supplemented as stated above by the general morphological relationships present.

The differentiation attained by the grafted tissues is of two kinds, cartilaginous and epithelial. No ganglion cells or other nervous tissue of any kind can be found in the grafts. This fact emphasizes the isolation of the primordium before its transplantation, for the ganglion and the otocyst are very closely associated at that early stage. The cartilage partially surrounds the epithelium, the latter forming the lining of cavities of various sizes. The relationships of the various parts of the organ is not normal. I have selected for description two cases which show quite an extensive specific differentiation of the membranous labyrinth.

Graft 32 *A* 5 is a growth obtained from the transplantation of a forty-eight hour primordium which has remained in the host membrane for six days, a total of eight days growth. Completely surrounding a large epithelial sac is a capsule of cartilage which is discontinuous in two places. Through one gap, small blood vessels enter a narrow group of mesenchymal cells which separate the epithelium of this region from the cartilage surrounding it. The other break in the cartilage sheath is in a place where an incompletely incorporated portion of the epithelium makes a connection with the epithelial sac mentioned above. The cartilage, from its general relations, is undoubtably the otic capsule.

The epithelium of the membranous labyrinth shows a high degree of specificity in its mode of differentiation. It may be divided into two main parts, that incompletely incorporated and outside of the cartilaginous capsule, and that within the capsule. The epithelium which is outside of the cartilage is histologically identical with that of the normal saccus endolymphaticus, the part connecting the saccus endolymphaticus with the epithelium of the capsule, the ductus endolymphaticus. These two portions

are surrounded by mesenchyme only, as in the control. The parts within the cartilaginous capsule show a high degree of differentiation also. In the region of the vascular foramen referred to above, the epithelium has the characteristics of the normal utriculus of the eight day chick embryo. This determination is further confirmed by the appearance in the epithelium of three thickened areas which represent the cristæ acusticæ of the ampullæ of the semicircular canals. No ganglion cells are associated with these as in the controls. There are no canals or ampullæ formed though at a point occupied by one of the cristæ (Fig. 7.

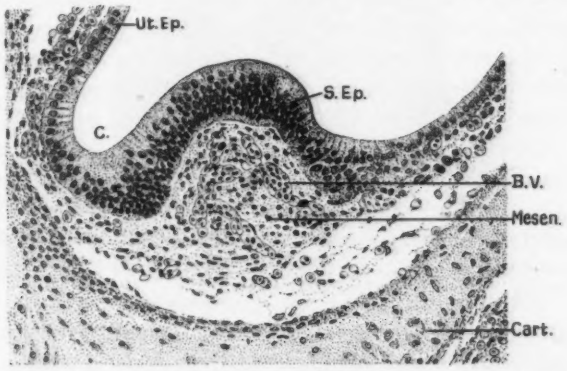

FIG. 7. Section of graft of otic vesicle of forty-eight hour chick in region of one of the cristæ acusticæ. Eight days growth, 32 *A* 5. (× 235.) *B.V.*, blood vessels. *C.*, evagination suggesting beginning of canal formation. *Cart.*, cartilage of otic capsule. *Mesen.*, mesenchyme containing blood vessels. *S.Ep.*, crista acustica. *Ut.Ep.*, utricular epithelium.

Compare Fig. 8) there is a slight evagination which suggests the beginning of such formation. It is very limited in extent, appearing for only a few sections. Through the space formed by the second break in the cartilage, the large sac lined by utricular epithelium is connected to the endolymphatic derivatives mentioned above. A few sections beyond this the wall of the sac shows a differentiation into a stratified sacculus-like and a thinner utricular epithelium. The ductus cochlearis and the lagena which normally arise from the sacculus are absent in the graft. It may very well be that the presence of the cartilage sheath has acted as a mechanical obstruction to their formation.

The same definite differentiations of the epithelium show well in case 40 *B* 6. Here, as in the case cited above, both cartilage and epithelium are present. The cartilage is not so extensive, however, but is located near, though not surrounding, the epithelial tissues. The relations of the parts of the membranous labyrinth are very much disorganized in this graft, the tissue

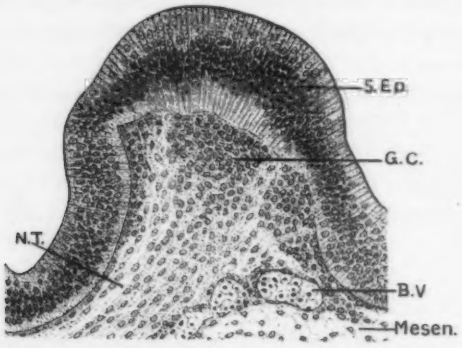

FIG. 8. Section of crista acustica of one of the ampullæ of a chick of eight days incubation. (× 235.) *G.C.*, ganglion cells. *N.T.*, nerve tract running from crista to brain. Other abbreviations as in Fig. 7.

appearing in two places, separated by the host mesenchyme. The character of the cellular differentiation is distinctive. In one of the groups, the differentiation is like that of the ductus and saccus endolymphaticus. These structures are connected, the ductus appearing as a tube leading from the saccus. Near these, a canal-like formation appears for a few sections These parts are not widely separated from the other group of structures which are adjacent to the mass of cartilage. The parts in this group are in the form of distinct vesicles and tubules, resembling histologically, utriculus, sacculus and a small portion of a canal. In the utricular wall are two areas interpreted as cristæ acusticæ, one of which is associated with the canal-like portion already mentioned.

It would seem therefore, from the data given for the above cases, that the differentiation of the constituent parts of the otic vesicle is very specific, and that it takes place independent of the relationships of the various parts, which depend on mechanical factors.

Streeter (1907, 1914), who has experimented extensively on the development of the ear vesicle in the amphibian larva, has noted the ability of the vesicle to develop in a nearly normal manner when transplanted to other portions of the head either of the same or opposite side. In most of the cases, it reorients itself as to dorso-ventrality and retains its definite character as from the right or the left side. In Spemann's experiments (1910), the inverted otocyst did not orient itself in every case, due, in Streeter's opinion, to the fact that in making the large incision for the rotation of the vesicle, the adjacent tissue was much disturbed and also to the factor of mechanical pressure which was applied to insure the closure of the wound. Streeter eliminated these factors by making one slit and stretching this for the operation. Closure and healing thus took place immediately without the application of pressure. Spemann's results do not otherwise seem to be in conflict with those of Streeter. The results of both of these investigators emphasize the high degree of independent self-differentiation obtained by the organ. Streeter notes (1914, page 172), "All our evidence points to a high degree of differentiation of the cells of the vesicle, and it is conspicuously proven by their possession of laterality, . . ."

It will be of interest to note also a case cited by Lewis (1907), in speaking of which he states that an otic capsule of the host, *Amblystoma*, formed around the membranous labyrinth arising from transplanted tissue from *Rana sylvatica*. He adds that the character of the cartilage cells is such as to determine them as of Amblystomal origin. Lewis considers that this shows that in some way, the otic vesicle stimulates the formation of the cartilaginous capsule around it. He says (page 145), "If the cartilaginous capsule about the otic vesicle is dependent upon the influence of the latter for its origin it will probably be found the cartilage of other regions of the embryo is likewise dependent on certain influences in the neighboring structures for its origin from the mesenchyme. . . . A piece of transplanted brain or a transplanted eye, for example, even when close to the cartilage forming about the otic vesicle or central nervous system does not stimulate the formation or growth of cartilage about itself."

In the grafts of the otic vesicle described above, the cartilage

is identified as that of the otic capsule but it is very definitely the result of the differentiation of the cartilage-forming tissue of the graft and not of the host mesenchyme stimulated by the membranous labyrinth. The very fact that it only partially surrounds the parts of the vesicle proves this. It will be recalled that a fringe of mesenchyme was transplanted together with the otic vesicle. This would easily account for any cartilage present in the grafts.

The specificity of the cellular elements of the otic vesicle is very marked (see also Streeter, *loc. cit.*). Utriculus, sacculus, endolymphatic duct and sac, and the canals are recognizable by the character of their histological differentiation. Though the canals have not formed in any of the cases, their general position is indicated by the occurrence of the cristæ acusticæ of their ampullæ. In no case are they accompanied by ganglion cells. Neither is the auditory nerve present in any of the grafts. This indicates a complete isolation of the primordium, for the eighth ganglion is closely associated with the otocyst even at the stage used in the operations. There is therefore no possibility of a stimulation of the epithelium of these sensory areas by nerve cells. These areas are localised in the epithelium itself. By means of various grafting experiments, Harrison (1903) found a similar differentiation of a sensory area, independent of nervous stimulation, to exist in the development of the lateral line organs of *Rana sylvatica* and *Rana palustris*.

In spite of the much altered relationships of the parts of the membranous labyrinth and the surrounding mesenchyme as they develop in the grafts, they differentiate so that they are easily identified in the sections. The amount of adjustment of the relationships of their parts is, on the other hand, very variable, being dependent on the amount of mechanical injury and the position of the grafted tissue at operation, together with the factors of pressure, and stress and strain during the following growth period.

D. The Mesencephalon.

As has been stated above, the selection of the mesencephalon in making grafts of brain tissue was made because of the fact that it is here that the correlation centers of the eye have their seat.

In the adult bird it forms the large corpora bigemina which give off no peripheral nerves.

In the embryo, at about the forty-eight hour stage, the brain wall in this region is sufficiently independent of the ectoderm to enable the isolation of this part of the brain with very little or no mechanical injury. It is comparatively easy to remove all of the metencephalon and the diencephalon from such pieces, so that the transplants contain only mesencephalon. Since such material yields grafts favorable for this study and since the injury at an earlier stage of development is of necessity much greater, the entire series of experiments with this primordium was made with tissue from chicks of forty-eight hours of incubation.

For the sake of comparison, a brief description of the mesencephalon of the eight-day chick will be given here. The whole region is divided into two portions, a basal and a tectal. These surround three connected cavities the larger ones being the mesocœles, and the smaller one, the aqueduct of Sylvius, which is within the basal portion of the region. The differentiation of the wall of the corpora bigemina, the tectum lobi optici, is very definite. If we examine a cross section of part of this region, we find that there are four cortical layers present (Fig. 9). These will be described in the order in which they are found on examination of such a section, from the ependyma to the periphery. Next the mesocœle is an ependyma which is densely nucleated and fairly wide. From it, cell bodies appear to be migrating toward the periphery. The next layer is an inner fiber zone which stands in great contrast to the nucleated zone just mentioned. It is rather wide and contains cords composed of the migrating cell bodies and their processes. The migration of these cell bodies ends in a nuclear layer which lies just peripherad to the inner fiber layer. The processes of these cells extend still further out, forming an outer fiber layer which is much narrower than the inner fiber layer and contains no nuclear strands. Bounding the outer fiber layer is a membrane, the external limiting membrane, on the outer side of which is a thin layer of mesenchyme containing blood vessels.

The outer fiber layer contains processes of the cell bodies of the outer nuclear zone. These are shown very clearly in tangential

sections of the lobe, in which fiber tracts appear, running for some distance across the field. Longer fiber tracts appear in the inner fiber layer. These run into the base of the lobes, through which all of the innervation of this region finds its exit. The basal por-

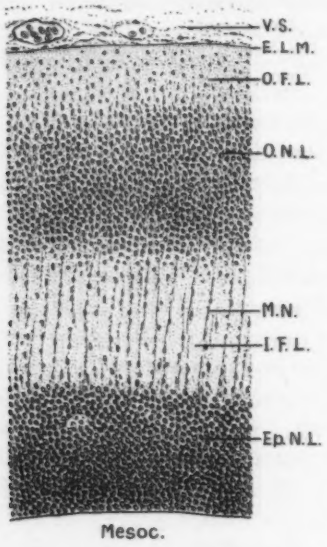

FIG. 9. Cross section of a portion of the tectal part of the mesencephalon of a chick of eight days incubation. (× 180.) *E.L.M.*, external limiting membrane. *Ep.N.L.*, ependymal nuclear layer. *I.F.L.*, inner fiber layer. *Mesoc.*, mesocœle. *M.N.*, nuclear strands. *O.F.L.*, outer fiber layer. *O.N.L.*, outer nuclear layer. *V.S.*, vascular sheath in the mesenchyme.

tion also contains fibers of the ascending and descending nerve tracts. No peripheral nerves are present. The vascularization of the region is accomplished by small vessels which enter the nervous tissue through the external limiting membrane.

Bearing this general condition in mind, an examination of the sections of graft 38 *A* 4 shows, with a few exceptions, a general conformability of its development to that of the normal. The two greater cavities of the control are represented by one large mesocoele. This is bounded by the basal and tectal portions of the grafted tissues. The basal portion gives a rather compact appearance with many fiber tracts. In the cortical region the

differentiation is very striking. The two nuclear layers are present and separated by the inner fiber layer which contains the nuclei in definite strands. In tangential sections this layer also shows many fiber tracts. In this case, this fiber layer also shows a very heavy infiltration of blood vessels, a condition not present in the normal. Such a condition seems to be correlated with

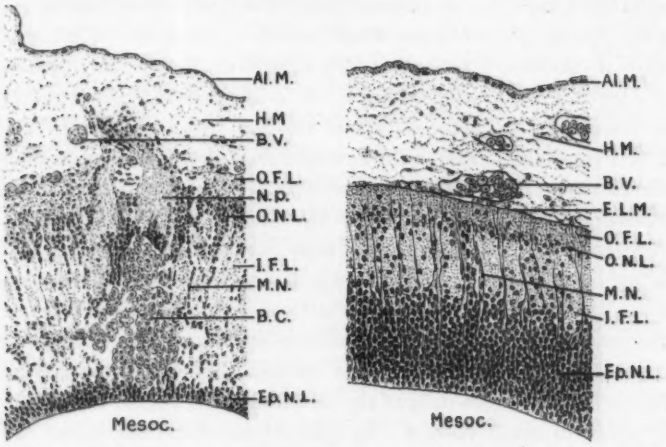

FIG. 10. Section of a portion of a graft of the mesencephalon of a forty-eight hour chick; external limiting membrane not present. 38 *A* 4. (× 180.) *Al.M.*, allantoic border of membrane. *B.C.*, blood corpuscles. *B.V.*, blood vessel. *H.Mesen.*, host mesenchyme. *N.P.*, nerve-like process. Other abbreviations as in Fig. 9.

FIG. 11. Cross section of a portion of a graft of the mesencephalon of a forty-eight hour chick; external limiting membrane present. 18 *B* 5. (× 180.) Abbreviations as in Figs. 9 and 10.

certain other differences in the development of the parts of the primordium, the most evident of which is in the outer fiber layer. In this graft (Fig. 10), the outer fiber layer is not bounded by an external limiting membrane and as a result the fibers penetrate the adjacent mesenchyme. These fibers have grown out into the mesenchyme, or the mesenchymatous tissue of the host has advanced into the nervous tissue of the graft; the width of the zone in which fibers alone are present is very variable. The outer layer may even be lacking in many places, the outer nuclear layer being in immediate contact with the loose mesenchyme

cells of the host, between which the nerve processes make their way. At numerous points along the periphery these processes are united into nerve-like masses which end bluntly at varying distances from their point of origin. Some of the cell bodies of the outer nuclear zone appear to have migrated through the immediately surrounding loose mesenchyme to a more definitely organized envelope of mesenchymal tissue which surrounds the graft and in which run the blood vessels of the host. The structure of this sheath resembles very closely that of the corresponding layer in the normal embryo. It is impossible to say whether it arose from the mesenchyme of the host or of the graft. The blood vessels are those of the host and they show a most extensive development. In places, large spaces filled with blood are present between this layer and the developing mesencephalon. Apparently because of the fact that the external limiting membrane is not present in such places, the vascular infiltration of the fiber layers as well as the nuclear layers is enormous. Even in the mesocœle there are large groups of blood cells though no organized vascular walls appear around them. In the nuclear layers there is a crowding of the developing nerve cells due to the presence of small vessels. In spite of this fact, the layers are distinct. The individual cells of the areas seem to be differentiating in a normal manner.

The significance of the absence of the external limiting membrane becomes evident upon examination of another graft, 18 *B* 5 (Fig. 11), in which this membrane is present around a majority of the cortical tissue. Where it is present, the differentiation of the layers of the cortex corresponds exactly with that of the control. In the peripheral fiber zone, the fibers form a clear band and do not extend into the mesenchyme. As a result, none of the nerve-like processes are found. The vascularization of such regions is much less extensive than that of regions where the membrane is absent. As a result of this, practically no distortion of the nuclear and fiber layers occurs. These are normally proportioned and contain cells which are in the process of normal differentiation.

In other portions of the same graft there are areas lacking the external limiting membrane. As we should expect, the outer

fiber layer is reduced or absent; processes of the nerve cells penetrate the mesenchyme, forming nerve-like structures; and the layers of the cortex are very heavily vascularized.

This graft shows a single large mesocœle as does the other case described. At one end of the sections of both grafts, where the roof of the mesencephalon is cut tangentially, extensive fiber tracts are visible in the inner fiber layer. The mesocœle in both cases is completely enclosed by nervous tissue, the brain having fused at both ends. This was aided in all probability by the apposition of the cut edges at section. The gross morphology of the organ arising from such a graft seems therefore to be affected to quite a considerable extent by the environment though the differentiation of its cellular constituents is specific.

The growth and differentiation of the mesencephalon in grafts made upon the chorio-allantoic membrane of the chick embryo brings out a number of very interesting facts. The basal and the tectal portions retain their individuality though the two cavities are blended into one large mesocoele. The basal portion develops more or less irregularly, dependent apparently on the mechanical factors involved and the absence of ascending and descending tracts when isolated. The tectal portion, however, shows two types of reaction, both of which have already been described. The interesting fact about the development of this part is found in the form taken by the outer fiber layer, dependent on the presence or absence of the external limiting membrane. This also affects the other layers inasmuch as this membrane appears to regulate the penetration of blood vessels from the host (cf. supra). The growth and the differentiation of the various layers is very specific in both cases. The nuclear cords and the width of the layers, with the exception of the outer fiber layer, as has been mentioned above, are approximately normal.

It was mentioned above that in some of the grafts of the mesencephalon and the nasal region, there are small peripheral nerve-like bundles present which do not correspond to any such formation in the normal. These outgrowths of nerve are the result of the differentiation of the individual fibers of the nerve cells, but, owing to the fact that the membrane which furnishes a mechanical obstruction to indefinite outward growth of

these is absent, these fibers have continued to grow into the mesenchyme, forming, in places, nerve-like bundles. Lewis (1907 *d*) describes a similar outgrowth of nerves from the brain tissue of *Rana sylvatica* from which he had removed the optic primordium. He states (page 464): "These nerves extend from a region of the brain which, under normal conditions, never gives rise to peripheral nerves, or at least to nerves that leave the brain in the region to run into the mesenchyme. . . . The injury in the brain in these experiments was done long before the nerves normally appear." The data presented here seem to indicate that the regulation of the growth of the elements is dependent to some degree on the structural environment, and that the disorganization produced by the injury during operation is sufficient to modify the gross structure obtained in development.

IV. Discussion

The independent power of growth and differentiation of other embryonic parts has been studied to a limited extent by this same method. Danchakoff (1921) has investigated the growth and the differentiation of the blastoderm of the "primitive streak stage" of the chick embryo after transplantation to the "allantoic" membrane. Blastoderms of "ten and more somites" from which the posterior portion to the eighth somite had been removed, were also grafted. In the blastoderms transferred to the allantois at an early primitive streak stage, no differentiation of organs appeared; the implanted parts remain as "islands of undifferentiated tissue." Growths from blastoderms of a late primitive streak stage showing a slight indication of the head process, show groups of cells which "grow well and reach a high degree of differentiation." Eye, nervous tissue, and notochord are present, and in the cartilage which forms around the cord, different parts of normal vertebræ are recognizable. Danchakoff concludes from her results, "the earlier a whole blastoderm is transferred to the allantois, the less growth and differentiation is obtained." While the principles involved in the growth and development of such transplantations are probably the same as those applying to the independent differentiation of the isolated organ primordium, the situation in grafts of such large portions

of the embryo is very complex and for this reason an analysis would be difficult.

More recently Atterbury (1923) has transplanted the metanephric "anlage" of the chick in the same way. The material used in the experiments was taken from seven day chicks and transplanted to the membranes of chicks of the same age. The primordium of the kidney consists of an ureteric channel with many lateral tubules and metanephrogenous tissue at the time transplanted. The differentiation obtained from such grafts after various periods of growth was similar to that of the control metanephros. The isolated metanephric primordium of the chick embryo has thus the power of independent self-differentiation as have the organs studied in this report.

A general survey of the power of growth and differentiation of isolated parts of the embryo was made as a preliminary to the present study. The grafts obtained from implants of cross sections of the somitic region and the primitive streak confirm the results that Danchakoff has obtained for the "blastoderm in toto" of the younger embryo. Cartilage, muscle, nervous tissue, nephrogenous tissue, and epidermis differentiate well. Some of the muscles of the somitic region show very definite striations. The cartilage suggests the form it would have assumed in the normal embryo and the spinal nerves have a serial arrangement indicating the segmentation in the cord.

If the data dealing with more or less extensive organ complexes are compared with the results obtained in the investigation of the isolated primordium of the single organ, it appears that in both cases, the differentiation of the graft is of a very specific nature, after a certain amount of physiological differentiation has taken place in the parts transplanted. This physiological differentiation of the cells of the organism has been called "protoplasmic specialization" by Child (1921). In speaking of "self-differentiation" in isolated parts of annelid and mollusk embryos he states (p. 120) ". . . protoplasmic specialization has advanced so far that the regulatory reactions to isolation of parts are limited, and the behavior of the part after isolation is therefore much the same as before." Just when the specialization of the cells of the organ primordium is established in the chick embryo is a matter requiring further investigation, though the results of

the present study indicate that it is at a very early stage. It is conceivable that, for the eye, for example, specialization may occur after the earlier stages used by Danchakoff, for in her experiments, the entire embryo with a part of the blastoderm and not the isolated primordium was transplanted, so that physiological isolation was not complete. The time of such organization differs for different animal classes as has been shown by the experiments on the independent development of isolated blastomeres, some cleavage being determinate from the very first with no post-regeneration. The antero-posterior course of embryonic differentiation in the chick indicates that the time of this specialization varies for different parts of the same embryo.

The results of the study of the organs described indicate that the cells of their parts possess a specificity which is histologically equal to that of the normal. That such a specificity is an innate property of the cells of the embryo at certain stages has, moreover, been shown by numerous investigators by the methods of tissue culture, where the cells behave more or less as independent units. The same principles apply to the development of the subordinate parts of the organs grafted. This is emphasized by the differentiation of the cristæ acusticæ of the membranous labyrinth, the lens and the lenticular zone of the eye, and the various parts of the nasal region and the mesencephalon. The development of a subordinate part when isolated as such, is a problem, the results of which must be established by further investigation. That such parts are capable of differentiation without the stimulation of external factors is proven by their behavior when isolated in the primordium of the organ. No nerve stimulation of the cristæ acusticæ is necessary for their origin, for example, a condition closely resembling that found to exist in the formation of the lateral line sense organs of the amphibian larva by Harrison. The results which have already been obtained indicate that the same general principles are involved in the differentiation of the parts of the other primordia which have been employed in the transplantations.

The limits of development of the isolated parts seem to depend on the mechanical factors involved rather than on the physiological isolation as such. In none of the cases is this more marked

than in those where the otocyst and mesencephalon are used in transplantation. Despite the disorganization produced by the manipulation and by the forces acting on the graft during its growth on the host, the results of the isolation are not marked, differentiation occuring in an approximately normal manner. The absence of the external limiting membrane of the mesencephalon, for example, does not alter the differentiation of the parts as such, but does modify the type of development obtained by the organ as a whole.

Bearing these points in mind, we may say that these results emphasize the mosaic type of the development of the primordia examined, after their origin in the embryo. The time of their origin varies, but once they are physiologically constituted as such, their isolation from the adjacent structures has little effect on the unfolding nature of the realization of the potentialities of their constituent parts. The limits which are imposed on the development of these transplanted organs are dependent on environmental conditions apparently largely of a mechanical nature. The nature of the development of the different cells is apparently very specific, but to what degree, remains the subject for further investigations which are being made at the present time.

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I wish here to express my thanks to Mr. Kenji Toda for the preparation of the figures contained in this report.

CYTOLOGICAL CHANGES IN UNFERTILIZED TUBAL EGGS OF THE RAT.

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INTRODUCTION.

Recent work on artificial parthenogenesis in vertebrates has reopened the question as to the possibility of some degree of parthenogenesis in mammals. Many authors have described division comparable to cleavage in eggs of atretic follicles, but no thorough-going study of the cytological changes in the tubal eggs of mammals has been made until recently. This lack of observation was natural as long as ovulation was thought of as occurring only at copulation, since under these circumstances the possibility that fertilization might have occurred could not have been excluded. Other work indicating that ovulation occurs at regular intervals in several mammals including the rat quite independently of copulation, lead to the question as to how far the cytological changes of the unfertilized tubal egg of the rat might parallel normal development. It was of interest to compare the changes in the unfertilized eggs in atretic follicles, as well as with eggs which have been artifically stimulated to parthenogenetic development. The conditions which have been described in these three cases are so much alike that one is inclined to believe that similar physiological changes are going on in each. Whether these processes are wholly disintegrative in the tubal rat egg, or represent an abortive beginning of parthenogenetic development appears to be a matter of interpretation. Several authors have stated, and this work corroborates the observation, that mammalian eggs disintegrate in the second polar spindle stage if fertilization does not occur soon after ovulation. Many other authors have noted the occurrence of divided eggs, simulating, at least, two, four, or more cell stages, in the lower portion of the oviduct and uterus of mammals. It is the purpose of this paper to trace the cytological changes which occur between ovulation and such apparent cleavage stages.

Material.

For the material used in the preparation of this paper I am indebted to Drs. J. A. Long and H. M. Evans. I also wish to express my thanks to Dr. Long for helpful encouragement. The material was prepared for a study of ovulation in the rat, *Mus norwegicus*. The females from which the ova were obtained were isolated before parturition and kept from males thereafter, thus preventing the possibility of ova being fertilized. They were killed at different intervals after parturition. The ovaries and oviducts were fixed in Zenker's or in Benda's fluid and stained in carmine, iron hæmatoxylin, or phosphotungstic acid hæmatoxylin. The latter was by far the most useful stain. The Benda material was especially valuable for study of the cytoplasmic elements, but it was less useful than the Zenker preparations for details of nuclear structure, because the darkly staining granules and mitochondria tend to obscure the chromosomes especially in the period of scattering. In the fifty oviducts which were studied eggs were found in the distal portion in twelve cases, in the middle upper portion in ten, in the lower middle portion in seven, and in the proximal portion in nineteen. In all two hundred and fifty-two eggs were examined. As many as nine eggs were found in some oviducts, but three, four, and five were noted much more commonly.

Observations.

At ovulation the second polar spindle lies parallel to the periphery of the egg with the dyads arranged upon it in typical final prophase position. In any one oviduct all of the ova are at about the same stage. Table I. demonstrates the fact that the commoner stages show a natural sequence with respect to position in the oviduct beginning with distortion of the spindle and scattering of the chromosomes and ending with the unequal fragmentation of a multinucleate cell. It is evident that we are thus provided with a natural seriation of cytological stages of great value in interpretation of the results.

Stage I.—It will be noted from Table I. that by far the greatest number of ova found in the distal third of the oviduct were in the second polar spindle stage. The spindle may, however, be slightly turned or even at right angles to its original position

parallel to the periphery. In a few cases some of the dyads have divided and the monads have moved apart from one another on the spindle fibers. First polar bodies are often found, but no evidence of second polar body formation was noted. The cytoplasm shows no marked differentiation, consisting of a rather coarse reticulum enclosing a few large yolk globules and many small granules. Some of them stain deeply and are found in chains. These may be mitochondria. The zona pellucida is

TABLE I.

SUMMARY OF CYTOLOGICAL DATA AS RELATED TO POSITION IN THE OVIDUCT.

Stage.	Series.	Position in Oviduct.[1]	Number of Eggs.	Cytological Notes.
	318	Distal	3	3 with second spindles slightly rotated in all.
	345	"	9	2 with second spindles parallel surface, 1 perpendicular; 6 with chromosomes scattered.
	383	"	4	4 with spindles parallel surface.
	337	"	3	1 with spindles parallel surface; 1 perpendicular; 1 with chromosomes condensed.
	227	"	3	2 with spindles parallel surface; slightly rotated in 1.
	10	"	4	4 with spindles parallel surface.
I.	103	"	7	Second spindles show some scattering, peripheral cytoplasm vacuolate.
	110	"	6	4 with spindles parallel surface; 2 show some scattering.
	111	"	4	4 with spindles parallel surface.
	18	2.5	2	2 with blunt spindles parallel surface.
	33	2.5	3	2 with 2d polar spindles parallel surface; 1 contains 1 large and 1 small spindle.
	113	3.5	6	5 with 2d polar spindles parallel surface; 1 central.
	102	3.5	1	1 with 2d polar spindles parallel surface.
	109	3.5	3	3 with 2d polar spindles parallel surface.
	115	4	9	5 with 2d polar spindles parallel surface; fragmentation in others; distortion.
	35	4	3	1 with 2d polar spindles parallel surface; 2 with spindles and vesicles.
	26	4.5	1	1 with chromosomes scattered, vesicles forming about them.
II.	22	4.5	2	2 with chromosomes scattered.
	367	4.5	3	3 with chromosomes scattered.
	107	5	3	1 with chromosomes scattered; 1 second spindle intact; 1 fragmenting.
	8	5	3	1 with chromosomes scattered, broken spindle; 2 second spindles.
	50	5.25	1	1 with chromosomes scattered, distorted spindle
	114	5.5	5	4 fragmenting, multinucleate; 1 with 3 large vesicles.
	347	5.5	7	6 with chromosomes scattered, 1 with 1 large group; 1 fragmenting.
	4	6	2	2 multinucleate.

Stage.	Series.	Position in Oviduct.[1]	Number of Eggs.	Cytological Notes.
III.	325	7	1	1 with 4 equal uninucleate cells.
	308	7	1	1 multinucleate, fragmenting.
	17	7.5	4	4 multinucleate.
	47	8.5	3	3 multinucleate.
	403	8.5	9	9 multinucleate, lobing irrespective of nucleus, fragmenting.
	12	8.5	3	2 multinucleate; 1 with spindle parallel surface.
	354	8.5	5	3 multinucleate and fragmenting; 2 with scattered chromosomes.
	42	8.5	2	2 multinucleate, fragmenting irregularly.
	453	8.5	9	9 fragmenting, with one or more nuclei; irregular.
	452	9	1	1 multinucleate.
	37	9	5	5 fragmenting with unusual regularity, 2 4 cells.
	314	9	?	Masses of uni- or multinucleate fragments.
	324	9	3	3 zona broken, fragments disintegrating.
	388	9	2	1 uninucleate cell; 1 group of 4 equal cells in a single row.
	48	9	1	1 with 4 multinucleate cells.
	32	9	5	5 multinucleate.
			152	

[1] The figures indicate the distance of the eggs from the oviduct; thus stage I. includes all eggs in the upper part of the oviduct, *i.e.*, from 1–3.5, while 9c is the region nearest the uterus.

Position in Oviduct.	Second Maturation Spindle Parallel Surface.	Second Maturation Spindle Slightly Rotated.	Second Maturation Spindle Perpendicular Surface.	Chromosomes Scattered.	Unfragmented Multinucleate Cells.	Fragmenting Multinucleate Cells.	Fragmented Ova.	Two Spindles in One Ovum.	Central Chromosomes Condensed.	2–4 Cell Stages.
1–3.5.....	34	4	2	15	0	0	0	0	2	
4–5.....	6	0	0	16	2	1	0	1	0	
5–8.5.....	1	0	0	7	14	13	0	1	0	1
8.5–9c....	0	0	0	2	0	6	22	0	0	2
Totals...	41	4	2	40	16	20	22	2	2	3

evident in all of the Benda and in some of the Zenker preparations as a wide homogeneous layer. Why it should have been preserved in some of the Zenker preparations and dissolved off in others is not understood. The follicle cells appear to be quite normal.

All of the eggs in twelve oviducts contained spindles either parallel to the periphery or somewhat perpendicular to it. In all, 47 eggs were found to be in this stage and of these forty were in the upper half of the oviduct.

Stage II.—In the upper middle portion of the oviduct the spindle is beginning to break down and the chromosomes are becoming scattered. In most of the unfertilized eggs studied the second maturation spindle remains parallel to the surface of the egg and there breaks down, the fibers splitting off and carrying the dumb-bell shaped chromosomes with them. This process varies in completeness in different ova. The spindle may be evident after most of the chromosomes are vesiculate, or it may disappear almost completely as the chromosomes scatter.

Eggs were found in the upper middle portion of the oviduct in ten cases, and in all forty eggs had broken spindles and scattering chromosomes. Thirty-one of these were in the upper half of the oviduct. The breaking down of the spindle results, at times, in a simulation of multipolar spindles but their irregularity and the fact that the chromosomes are usually widely scattered indicate that they result from the breaking down of a single spindle. Sometimes the spindle, when quite degenerate, is no longer fibrillar but appears as a dense homogeneous mass of material of a very distorted shape.

In two oviducts eggs were found in which the spindle was central, and the cytoplasm about it unusually dense. The spindle appeared shrunken and the chromosomes had coalesced into a compact mass. Such eggs were also found well down in the oviduct.

Stage III.—The vesicle formation which began in Stage II. proceeds until multinucleate cells are formed. A liquid gathers about a single chromosome or a group of them. The chromosomes then swell and break up into large and finally into small granules so that the nuclei assume the typical resting condition. They vary greatly in size and number, different cells in the same oviduct containing from one to ten nuclei which differ greatly in size. The appearance of the nuclei differs with the fixative used. In eggs fixed in Benda the chromatin lines the inside of a clear vesicle, most of it being in a mass on one side. In eggs fixed in Zenker the chromatin appears as granules on a net. Central spindles in different stages of degeneration were found in three oviducts. In one of these one egg contained a central spindle while the other three resembled typical resting cells. In the

other two, five eggs contained degenerating spindles while the rest were in an advanced stage of fragmentation.

Cracks often appear in the cytoplasm but the zona is still usually intact at this stage. The follicle cells are often grouped into small syncytia containing many small crescent shaped nuclei.

The eggs of stage three were mostly found in the lower middle portion of the oviduct. In all thirty-six ova were multinucleate. Of these sixteen were unfragmented, and twenty were beginning to fragment.

Stage IV.—Fragmentation begins almost simultaneously with vesicle formation but is usually completed in the most proximal portion of the oviduct. The fragments vary in number from two or three to many. They may be approximately equal in size or very unequal. A fragment may contain one to several nuclei or none. Fragmentation occurs quite independently of the vesicles when many are present. It may take place either by a process of lobing, or, more commonly, by the appearance of cracks in the cytoplasm.

Some of the ova in the lower portion of the oviduct resemble 2, 3, and 4 cell stages very closely. Three two, one three, and two four cell stages were found, In oviduct 32 only one egg in five resembled a normal condition, the rest being multinucleate and more or less fragmented. In oviduct 37 two two and one four cell stage were found, while two eggs were multinucleate and fragmented. Number 325 contained very degenerate fragments and one four cell stage. The cytoplasm is very degenerate in most of the fragmenting eggs. A zona may or may not be present but if it appears at all it is nearly always ruptured. The fragments are usually quite devoid of covering far down in the tube, and it is impossible to tell how many ova they come from. The follicle cells are now empty shells devoid of protoplasm.

Complete fragmentation was the rule in nineteen oviducts. All of these were found in the lower third of the tube. In all twenty-two ova had reached this stage but this number does not represent the total since in several cases degeneration had proceeded so far that the figures for them had to be omitted.

Literature and Discussion.

The observations upon atretic and unfertilized tubal eggs of mammals agree essentially but the interpretations are rather various especially with regard to atretic eggs. This is easily comprehensible since no natural method of seriation is provided in atretic material. On the other hand one can easily determine the position of tubal eggs in the oviduct, and as these data show, can then demonstrate that such eggs pass thru a regular series of changes as they descend the duct.

In eggs which are liberated into the fallopian tube the second maturation spindle sometimes rotates to a position at right angles to the surface. The dyads also divide occasionally so that the second anaphase really appears to begin in some unfertilized eggs. In by far the greatest number of eggs in the upper third of the oviduct, however, the second polar spindle is parallel to the periphery and intact, with the dyads still in late prophase position. The spindle may or may not be somewhat broken. No cytoplasmic changes were noted at this stage. In eggs in two oviducts the spindle was central in position.

Three of the facts cited above might be interpreted as indicating that the cell processes may not all be wholly degenerative at this stage. Firstly, the spindle may rotate as if in preparation for the second polar division. Secondly, some of the dyads may divide, and thirdly, the spindle may be found in the center of the egg. Eggs with central spindles all showed one of two degenerative conditions; the chromatin simply clumping into a single mass and the cytoplasm shrinking and becoming denser, or in other cases the cell wall may break and the very vacuolate cytoplasmic contents scatter. There is no indication in the tubal rat material that normal cleavage ever occurs in these eggs altho it may of course be possible that more material might include such stages since a few eggs give the appearance of approximately normal early cleavage stages. In by far the greatest number of eggs degeneration begins with the failure of the second polar division. It is of some interest that the formation of but one polar body is a characteristic feature of development of most naturally parthenogenetic animal eggs.

Degeneration has been shown to begin with the breaking of the

spindle and the consequent scattering of the chromosomes throughout the cytoplasm. Kingery ('13) described the same type of degeneration for atretic eggs of the mouse. The cytoplasm and chromosomes interact so that the latter at first appear to be surrounded by a clear liquid. They then swell and form little vesicles resembling nuclei. Where several chromosomes are contiguous large vesicles result. Charleton ('17) described the nucleus of tubal mouse eggs as forming from the entire chromosome group, which then gives rise to a multinucleate cell by lobing. The seriation here described demonstrates that this is not the case in the rat. If several contiguous nuclei were fusing, pictures such as Charleton has described as due to lobing would be obtained. Wilson ('01) has described syncytia in artificially parthenogenetic Toxopneustes eggs as resulting from a failure of cytokinesis following nuclear division by a mitotic process. The common appearance of broken spindles and scattered chromatin in the upper part of the oviduct as well as the difference in size of the vesicles indicates strongly that this is not the case in tubal rat eggs.

No true multipolar spindles, monasters or cytasters such as were described by Henneguy ('94, '95) for atretic rat eggs, by Newman ('13) for atretic eggs of the armadillo, and in artificially parthenogenetic Toxopneustes eggs by Wilson ('01) were found. One egg showed two partial spindles at opposite poles of the cell. Lams ('13) interpreted a similar condition in a tubal egg of the guinea-pig as indicating that the first polar body had not been detached from the egg, but notes that a similar case could not be so interpreted since the first polar body was clearly present. In view of the fact that the second polar spindle of tubal rat eggs sometimes snaps in two after great elongation and distortion it seems best to consider such pictures as originating in this manner. During the scattering of the chromosomes the cytoplasm becomes more coarsely granular and the meshes more open. Wilson ('01) described the cytoplasm of *living* artificially parthenogenetic eggs as becoming coarser by clumping of microsomes preceding aster formation.

One cell was found with one, and one with two resting nuclei. They are probably the result of chance grouping of chromatin

into one or two rather than into many masses.[1] When few nuclei are present they tend to occupy the center of the egg. Eggs containing resting nuclei were lower in the oviduct than any of the eggs containing spindles, hence it is probable that spindles never form following the disintegration of the second polar spindle in tubal rat eggs. Newman ('13) interprets his atretic armadillo material as indicating that new mitotic figures with asters form following a return to the resting condition after the failure of the second polar division, but none of the rat eggs with central spindles showed any indication of astral radiations.

There is no indication in the rat material that fertilization by a polar body ever occurs. The first polar body is completely separated from the egg before ovulation takes place.

Fragmentation occurs almost independently of the newly formed "nuclei," so that some of the resulting cells contain nuclei and some do not. Newman ('13) and Charleton ('17) describe a similar cutting off of anucleate portions of the egg and consider it as a form of deutoplasmolysis. Newman believes that such a process would be advantageous to a cell system governed by a haploid nucleus. Since in the rat eggs the distribution as well as number and size of the "nuclei" is haphazard in the fragments it seems improbable that the process should be considered normal. In the tubal rat material it appears that the fragmentation process is less abnormal when few nuclei are present in the egg, and that it may at times be fairly normal is indicated by the fact that a few normal-looking 2, 3, and 4 cell stages were found. In his observations on unfertilized eggs of Toxopneustes Wilson ('01) states that most of the blastomeres resulting from a simultaneous cleavage of syncytia contain nuclei but that some do not. Here also the fewer nuclei present, the more normal the division. Thus in tubal rat eggs as in artificially parthenogenetic ones, a few may have more vitality than the rest, and these may possibly be considered as possessing a very limited parthenogenetic capacity.

In the lowest portion of the oviduct all of the ova were obvious-

[1] The possibility that sperm may live in the oviduct for several days and then be capable of fertilization should, however, be taken into consideration in interpreting these occasional normal looking cases. Wilson ('01) also found some unfertilized eggs which resembled fertilization stages.

ly degenerate. They might be expected to enter the uterus, if at all, as mere shells and scattered cytoplasm. The zona is either broken or absent at this stage.

Conclusion.

Following ovulation most of the changes undergone by tubal rat eggs are distinctly abnormal. The central position of the spindle, the occurrence of three uni-, and one binucleate cell, the coarsening of cytoplasmic structure, the observation that division is more or less regular according to the number of "nuclei" which are present, and the discovery of a few two, three and four cell stages may be thought to indicate some tendency towards normal development in certain of the unfertilized eggs. On the contrary, the fact that the central spindle is rare and always degenerate, that only three uninucleate cells were found, the great preponderance of multinucleate cells, and the fact that only very degenerate fragments are found near the uterus, make it necessary to conclude that although certain auto-regulative processes may occur in a few ova, a definite type of degeneration is the rule.

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